Attorney's Docket No. 1034284-000001

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of	)	
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Blas FRANGIONE	)	Group Art Unit: 1649
	)	
Application No.: 10/540,294	)	Examiner: Kimberly A Ballard
	)	
Filed: January 26, 2006	)	Confirmation No.: 8280
	)	
For: METHOD FOR TREATING AMYLOID DISEASE	)	

SUBMISSION UNDER 37 CFR 1.99
INFORMATION DISCLOSURE STATEMENT

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

The accompanying information is being submitted in accordance with 37 C.F.R. § 1.99.

Pursuant to 37 C.F.R. § 1.99, a copy of each of the documents cited is enclosed.

To assist the Examiner, the documents are listed on the attached form PTO-1449. It is respectfully requested that the Examiner consider each of these references during substantive examination of the referenced case.

The fee set forth in 37 C.F.R. 1.17(p) is enclosed.

A copy of this submission is being served upon applicant pursuant to 37 C.F.R. § 1.248 by first class U.S. mail. Proof of service, including the date and manner of service, is attached.

This submission does not include any explanation of the patents or publications or any other such information. The submission cites no more than ten total patents or publications.

The submission is being filed within two months of the date of publication of the application (§ 1.215(a)) or prior to the mailing of a notice of allowance (§ 1.311), whichever is earlier.

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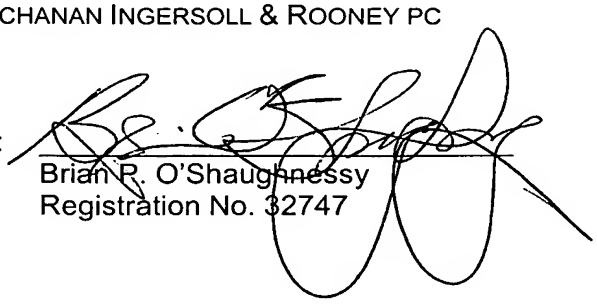
WILLIAM R. DIXON, JR.
SPECIAL PROGRAM EXAMINER

The Director is hereby authorized to charge any appropriate fees under 37 C.F.R. § 1.16, 1.17 and 1.21 that may be required by this paper, and to credit any overpayment, to Deposit Account No.02-4800. This paper is submitted in duplicate.

Respectfully submitted,

BUCHANAN INGERSOLL & ROONEY PC

Date: March 9, 2007

By: 

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Substitute for form 1449/PTO & 1449B/PTO

Complete if Known

Application Number	10/540,294
Filing Date	January 26, 2006
First Named Inventor	Blas Frangione
Examiner Name	Kimberly A. Ballard
Attorney Docket No.	

INFORMATION DISCLOSURE STATEMENT UNDER 37 CFR 1.99

(use as many sheets as necessary)

Sheet 1 of 1

U.S. PATENT DOCUMENTS

Examiner Initials	Document Number	Kind Code (if known)	Name of Patentee or Applicant of Cited Document	Issue/Publication Date (MM-DD-YYYY)
	US 2002/0009445	A1	Du et al.	01-24-2002

FOREIGN PATENT DOCUMENTS

Examiner Initials	Document Number	Kind Code (if known)	Country	Date of Publication (MM-DD-YYYY)	STATUS						
					Translation	Partial Translation	Eng. Lang. Summary	Search Report	IPER	Abstract	Cited in Spec
	WO 98/39653		PCT	09-11-1998							
	WO 03/051374	A2	PCT	06-26-2003							

NON-PATENT LITERATURE DOCUMENTS

Examiner Initials	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.
	E. Carro et al., "Serum Insulin-Like Growth Factor I Regulates Brain Amyloid-β Levels" *Nature Medicine*, **8**:12 (Dec. 2002), 1390-1397, Nature Publishing Group
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Examiner Signature		Date Considered	

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with M.P.E.P. § 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to Applicant.

PROOF OF SERVICE

The undersigned hereby certifies that the attached Submission Under 37 CFR 1.99 is being served on March 9, 2007 on Darby & Darby by first class mail as follows:

Darby & Darby P.C.
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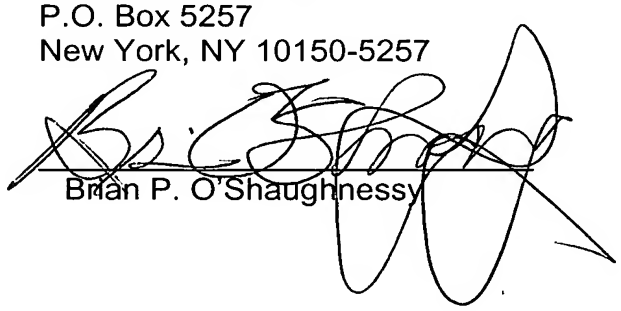
Date: March 9, 2007



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Du et al. (43) **Pub. Date: Jan. 24, 2002**

(54) **HUMAN BETA-AMYLOID ANTIBODY AND USE THEREOF FOR TREATMENT OF ALZHEIMER'S DISEASE**

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Publication Classification

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(52) **U.S. Cl.** **424/142.1**; 530/388.15

(57) **ABSTRACT**

The present invention provides according to the first aspect thereof a human anti-β-amyloid antibody obtained by purification from a human IgG-containing bodyfluid by Aβ-affinity chromatography. In a second aspect the invention provides a method of purification of an anti-Aβ-amyloid antibody, said method comprising the steps of obtaining a human IgG-containing bodyfluid, subjecting the bodyfluid obtained to an Aβ-affinity chromatography, and recovering the purified anti-Aβ antibody from the chromatography medium. Finally the invention provides for use of the above anti-Aβ antibody for diagnosing and/or treating amyloid associated diseases, especially Alzheimer's disease and for a pharmaceutical composition comprising said antibody for treatment of Alzheimer's disease.

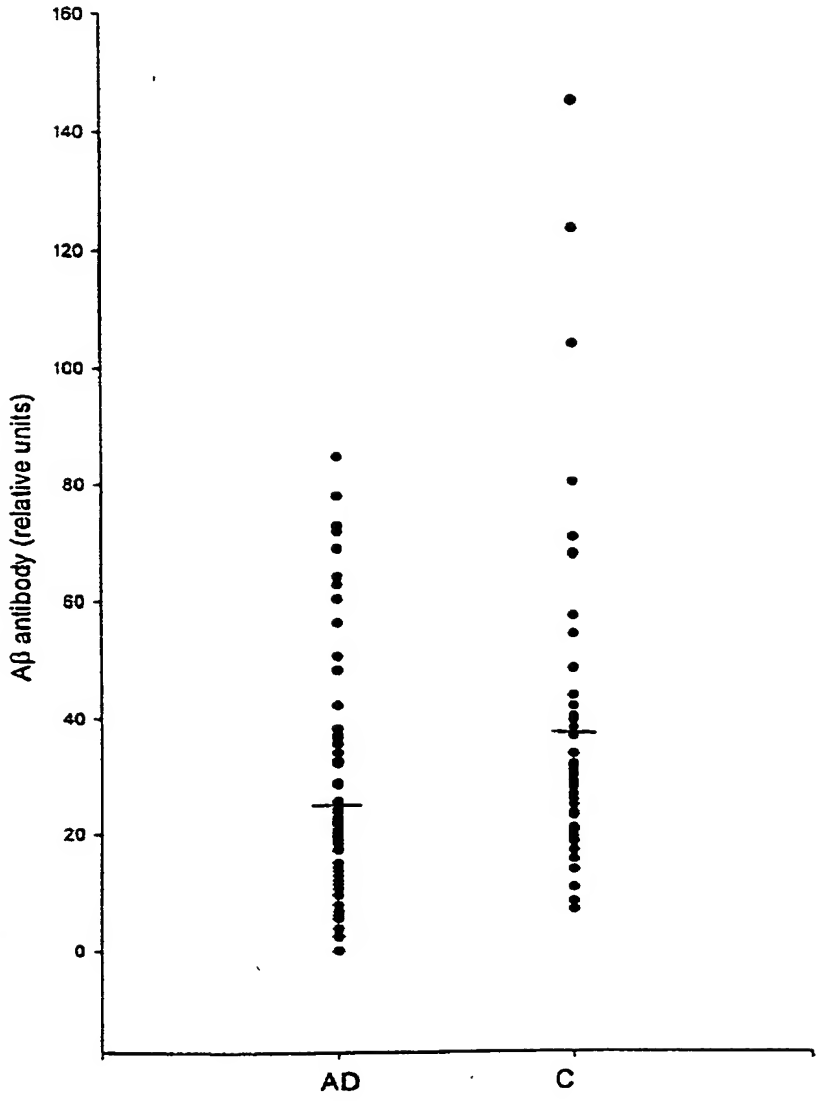
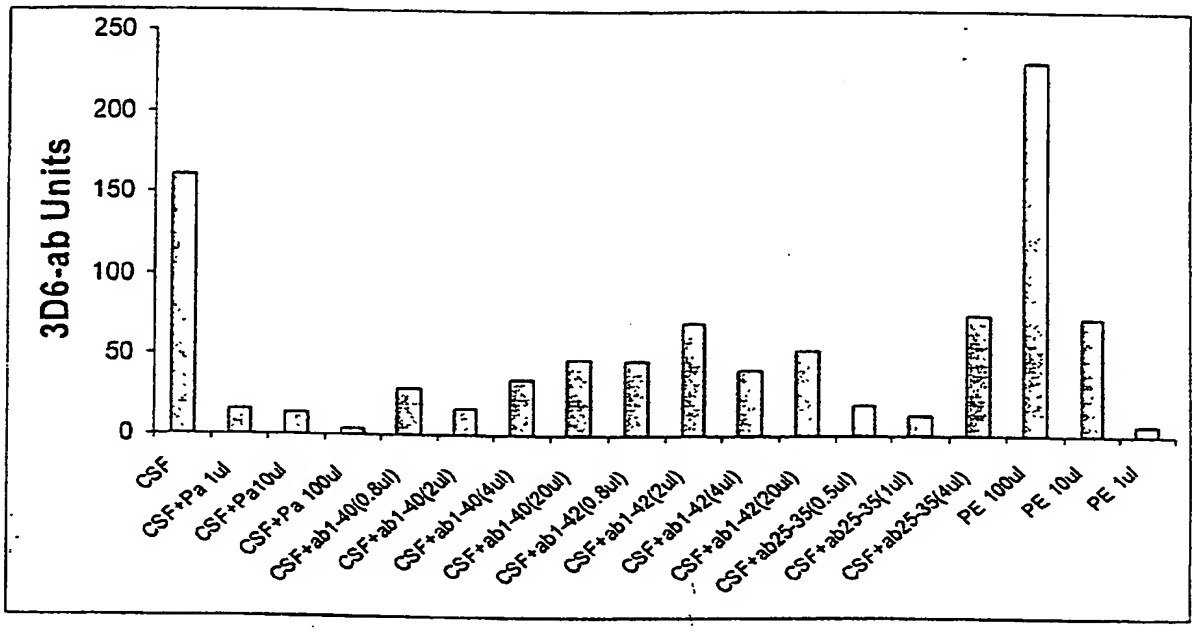


Figure 1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100
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CSF+ab1-40(0.8ul)
CSF+ab1-40(2ul)
CSF+ab1-40(4ul)
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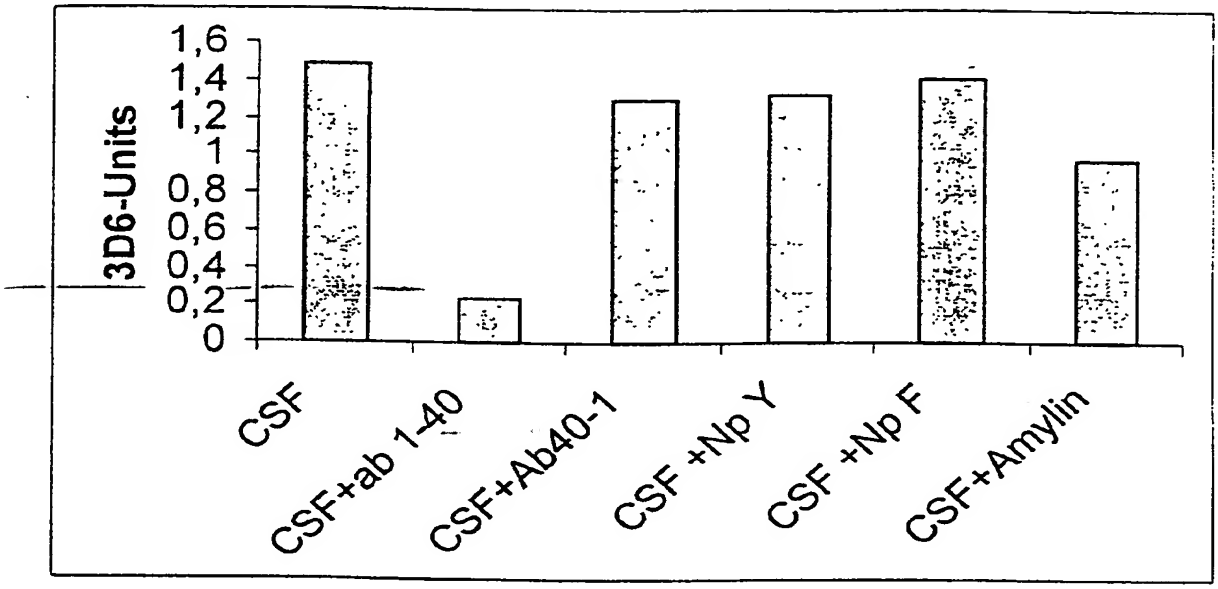
PE 10ul
PE 1ul

Figure 2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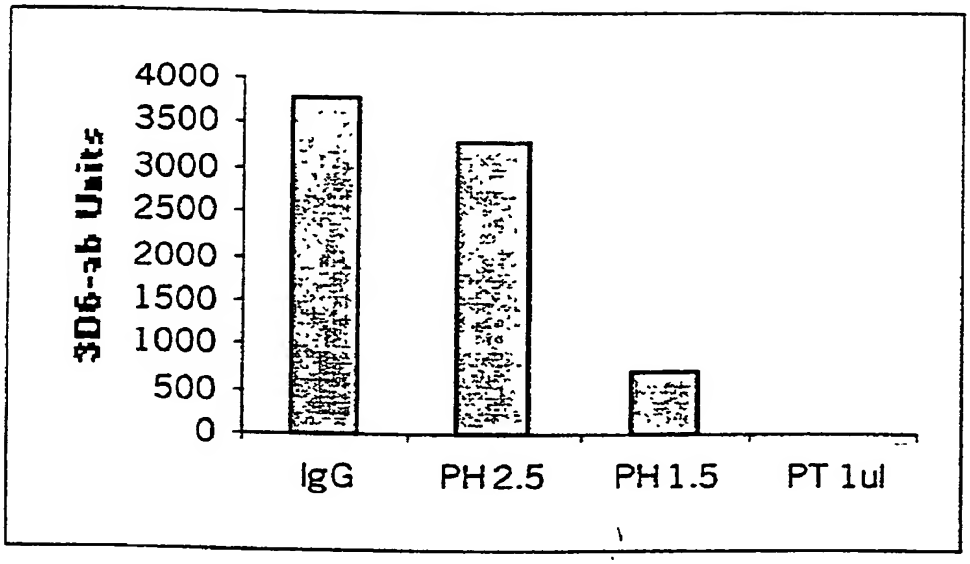
CSF+Ab40-1
CSF +Np Y
CSF +Np F
CSF+Amylin

Figure3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PH1.5
PT 1ul

Figure 4

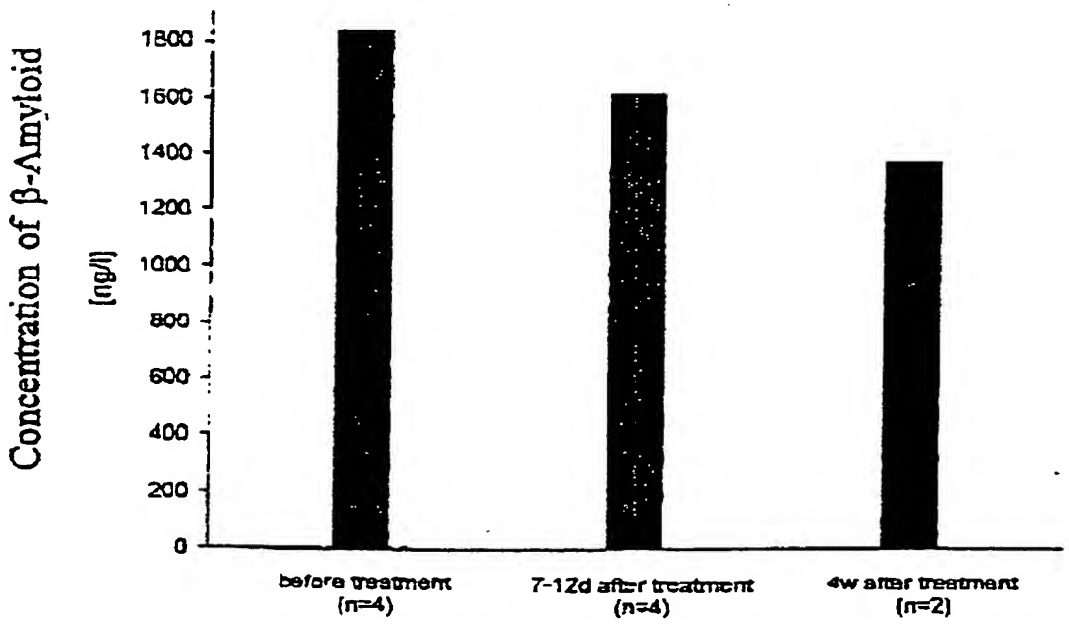
Concentration of β-Amyloid
[ng/l]
1800
1600
1400
1200
1000
800
600
400
200
0
before treatment
(n=4)
7-12d after treatment
(n=4)
4w after treatment
(n=2)

Figure 5.

HUMAN BETA-AMYLOID ANTIBODY AND USE THEREOF FOR TREATMENT OF ALZHEIMER'S DISEASE

[0001] The present invention relates to a human β-amyloid antibody, a method of purification thereof and the use of this ββ-amyloid antibody in treatment of amyloid associated diseases, especially Alzheimer's Disease.

BACKGROUND

[0002] Alzheimer's disease is a progredient disease initially manifesting itself with partial amnesia, and later restlessness, dysorientation, aphasia, agnosia or apraxia (cognitive decline), dementia and sometimes euphoria or depressions. The disease typically starts at 40 to 90 years of age and predominantly affects females. As to its occurrence, estimations are about 5% of the population above 65 years age. Alzheimer thus constitutes a major problem in industrialised countries.

[0003] In Alzheimer's disease brain region-specific amyloid deposition is a key neuropathological feature which is accompanied by astrogliosis, microgliosis, cytoskeletal changes, and synaptic loss. These pathological alterations are thought to be linked to the cognitive decline and dementia which defines the disease. These neuritic depositions or plaques and neurofibrillary tangles comprise the major neuropathological changes associated with Alzheimer's disease. Although other neuropathological changes have been linked to Alzheimer's disease, evidence indicates that they are as well somehow related to the classical lesions.

[0004] Neuritic plaques are spherical, multicellular lesions that are usually found in moderate or large numbers in limbic structures and association neocortex. The plaques contain extracellular deposits of β-amyloid protein (Aβ)that include abundant amyloid fibrils intermixed with non-fibrillar forms of this peptide. The major protein constituent of plaques is the β-amyloid protein (Aβ). Neuritic plaques have degenerating axons and dendrites within and intimately surrounding the plaque. Such plaques also contain variable numbers of activated microglia that are often situated within and near the fibrillar amyloid core, as well as reactive astrocytes surrounding the core.

[0005] The major constituent of the plaque, the β-amyloid protein, arises from a larger precursor protein, the amyloid precursor protein (APP). The amyloid precursor protein (APP) refers to a group of ubiquitously expressed proteins whose heterogeneity arises from both alternative splicing and posttranslational processes. Cleavage of APP in its COOH-terminal region in the transmembrane domain by β-secretase and γ-secretase results in the formation of the β-amyloid protein.

[0006] Aβ is secreted continuously by normal cells and can be detected as a circulating peptide in the plasma and cerebrospinal fluid (CSF) of healthy humans. In Alzheimer's disease it is thought that increased production of Aβ and/or a decreased metabolism of Aβ may lead to plaque deposition and consecutively to the neuropathological changes associated with Alzheimer's disease. Evidence for the role of Aβ in Alzheimer's disease include the observation that missense mutations in the APP have been found to be the cause of familial Alzheimer disease cases.

[0007] Several endogenous substrates, including apolipoprotein E have been shown to be associated with plaque formation. In transgenic mice APP^{V717F} (PDAPP) the lack of the apolipoprotein E gene (apoE-knock-out mice) results in the absence of amyloid plaque deposition (Games et al., Nature 1995; Bales et al., Nat Genet 1997). These transgenic mice (PDAPP) normally develop amyloid plaques in an age-dependent manner starting at three months of age.

[0008] Schenk and coworkers (Schenk et al, Nature 400:173, 1999) investigated the plaque burden in the PDAPP-mice following an immunization treatment. PDAPP-mice were immunised with pre-aggregated Aβ for different time periods using Freud's adjuvant. Plaque deposition in these mice decreased significantly following the immunization treatment. Sham-mice did not show a decrease in plaque deposition.

[0009] Treatment of APP^{V717F} transgenic mice with antibodies raised against Aβ was also reported to attenuate amyloid plaque formation, neuritic dystrophy and astrogliosis in younger mice as well as to decrease plaque burden in older mice. However, the finding could not be verified in other mice.

[0010] Despite of the above knowledge no therapy for amyloid associated diseases, especially Alzheimer's disease is available up to today. However, an effective therapy for Alzheimer's disease would be highly desirable because of its broad spread occurrence.

[0011] It is therefore an object of the present invention to provide such therapy of and/or means for diagnosing amyloid associated diseases, especially Alzheimer's disease.

SUMMARY OF THE INVENTION

[0012] The above object can be solved by a human anti-β-amyloid antibody and a pharmaceutical composition comprising the same as stipulated in the appending claims.

[0013] More in detail the present invention according to the first aspect thus provides a human anti-β-amyloid antibody obtained by purification from a human IgG-containing bodyfluid by Aβ-affinity chromatography.

[0014] In a second aspect the invention provides a method of purification of an anti-Aβ-amyloid antibody, said method comprising the steps of obtaining a human IgG-containing bodyfluid, subjecting the bodyfluid obtained to an Aβ-affinity chromatography, and recovering the purified anti-Aβ antibody from the chromatography medium.

[0015] Finally the invention provides for use of the above anti-Aβ antibody for diagnosing (with a special developed ELISA) and/or treating amyloid associated diseases, especially Alzheimer's disease and for a pharmaceutical composition comprising said antibody for treatment of amyloid associated diseases, especially Alzheimer disease and manufacture thereof.

DETAILED DESCRIPTION OF THE INVENTION

[0016] The applicants have now found that naturally-occurring Aβ antibodies exist in biologically relevant fluids i.e. CSF and plasma, and that levels of these antibodies differ between normal age-matched healthy controls and AD patients. Based on these findings it was concluded and then supported by experiments that the antibody can be used for diagnosis and treatment of amyloid associated diseases and

especially of Alzheimer's disease. In the context of this specification the terms "anti-Aβ antibodies" and "Aβ antibodies" are used interchangeably to designate the antibody of the invention.

[0017] In lumbar CSF samples which included 49 age-matched non-demented individuals with no family history of cognitive impairment and 60 individuals with confirmed AD, detection of CSF Aβ antibody levels was determined utilizing an ELISA assay in which the Aβ peptide was used as the capture ligand (see below).

[0018] Human anti-Aβ antibody was detected in CSF samples in both of the populations studied. It was confirmed that the Aβ antibody activity detected by the ELISA represents antibodies specific to Aβ by absorbing the activity with protein-A Agarose, and $A\beta_{1-40}$ or $A\beta_{1-42}$. However, no interaction was found between this Aβ-antibody and $A\beta_{40-1}$ or unrelated neuropeptides such as neuropeptide F or neuropeptide Y. The mean level of Aβ antibody in the Alzheimer's disease group was 30% lower than controls (control: 370±39, AD: 276±27; $p<0.05$, one way ANOVA).

[0019] These data demonstrate that an antibody directed against Aβ (anti-Aβ antibody or short: Aβ antibody) is present in physiologically relevant concentrations in human fluids, like CSF and serum. Antibody titres are significantly higher in control subjects than AD patients. The generation of naturally occurring Aβ-antibodies and subsequent Aβ/antibody complex formation, may be involved in the normal clearance of Aβ peptide(s), which serves to reduce Aβ deposition and neuritic plaque formation.

[0020] The lower titres of Aβ antibody found in more than 50% of the AD patients investigated in this study compared to controls suggest that reduced Aβ antibody generation and/or complex formation contributes to an abnormal (i.e. reduced) clearance function. Similar clearance problems may occur in other neurodegenerative diseases or amyloid associated diseases such as primary and secondary amyloidoses. The present invention thus pertains to treatment and diagnosis of these other amyloid associated diseases as well.

[0021] Based on the above hypothesis the treatment with antibodies against Aβ i.e. Aβ antibodies is a new strategy to treat diseases associated with amyloid deposition. These treatments include the increase of Aβ-antibody levels by using immunoglobulins (IgG), preferably human IgG with high titres of Aβ antibodies or using anti-Aβ antibodies purified from human IgG containing fluids. The present invention also encompasses use of antibody fragments (Fab etc.) as long as complex formation can be achieved.

[0022] Thus, the present invention relates to a human anti-β-amyloid antibody (Aβ-antibody) obtained by purification from a human IgG-containing bodyfluid by Aβ-affinity chromatography. Preferably the human anti-Aβ antibody belongs to the class of immunoglobulines G (IgG) and does not recognize $A\beta_{40-1}$, neuropeptide F, neuropeptide Y, and Amylin, and specifically recognizes one or more of $A\beta_{1-40}$, $A\beta_{1-42}$, and $A\beta_{25-35}$, and preferably recognizes all of $A\beta_{1-40}$, $A\beta_{1-42}$, and $A\beta_{25-35}$.

[0023] According to a second embodiment the present invention relates to a method of purification of an anti-Aβ-antibody comprising the steps of obtaining a human IgG-containing bodyfluid, subjecting the bodyfluid obtained to an Aβ-affinity chromatography, and recovering the purified anti-Aβ antibody. Preferably the IgG-containing bodyfluid is a fluid selected from the group consisting of cerebrospinal fluid, plasma and urine, all of them obtained from one or more human beings (pooled samples).

[0024] Furthermore, it is preferred that the Aβ-affinity chromatography is carried out by an Aβ-affinity column, obtained by conjugating $A\beta_{1-40}$ onto Sepharose 4B, elution with elute buffer at pH 1.5 to 2.5 at 4° C. using an FPLC system.

[0025] The present invention also relates to the use of the above anti-Aβ antibody and/or the use of an IgG containing, preferably IgG enriched fluid for diagnosing and/or treating amyloid associated diseases, especially Alzheimer's disease. Preferably the use is for treatment of amyloid associated diseases, especially Alzheimer's disease.

[0026] According to another embodiment there is provided a pharmaceutical composition comprising the anti-Aβ antibody of the present invention. A pharmaceutical composition of the invention comprises the anti-Aβ antibody and is preferably for parenteral administration, e.g. by i.v., i.m. or i.c. injection. It may comprise conventional carriers. A preferred dosage for administration is in the range of 0.001 to 3 g/kg body weight per day, a more preferred dosage for administration being in the range of 0.01 to 0,4 g/kg body weight per day.

[0027] The experimental work forming the basis of the present invention was carried out using the following materials and methods:

[0028] Aβ antibody ELISA:

[0029] 1 mg $A\beta_{(1-40)}$ is dissolved in 2 ml H_2O. Then add up to 200 ml coating buffer (1.7 mM $NaH_2PO_4*H_2O$; 98 mM $Na_2HPO_4*7H_2O$, 0.05% sodium azide; pH 7.4). Add 100 μl/well of coating buffer overnight at 4° C. Remove coating buffer and block plate with blocking buffer for 80 min. (blocking buffer 1: 0.25% casein in PBS, 0.05% sodium azide, pH=7.4). Wash plate 3 times with washing buffer (1×PBS/0.05% Tween-20). Load samples overnight at 4° C. Remove samples and wash plate 3 times. Add monoclonal anti-human biotinylated IgG in blocking buffer 1 for 1 h. Wash 3 times with washing buffer. Load antibody against biotin conjugated with horse radish peroxidase for 1 h. Wash four times and add TMP for 10 min, then add H_2SO_4 (1N) to stop reaction and read at a plate reader at 450 nm.

[0030] β-Amyloid-ELISA:

[0031] For the measurement of Aβ a commercially available kit for $A\beta_{1-42}$, $A\beta_{1-40}$ and $A\beta_{1-5}$ was used.

[0032] Cerebrospinal fluid (CSF) and plasma:

[0033] lumbar CSF and plasma were collected following standard clinical procedures after informed consent of the patients.

[0034] Criteria for the diagnosis of Alzheimer's disease:

[0035] All normal controls had no significant decline or impairment in cognition on clinical examination. They had no history or evidence of neurological disease with potential to affect cognition and no deficits in their ability to adequately perform activities of daily living (ADLs). All AD patients had a clinical examination, including neuropsychological testing, to document deficits in cognition and ADLs,

laboratory studies and a neurological examination to exclude reversible causes of dementia. All patients met ICD-10 criteria for dementia as well as NINCDS-ADRDA criteria for probable or possible AD.

DESCRIPTION OF THE DRAWINGS

[0036] **FIG. 1**: A-antibody has been identified in the CSF from Alzheimer's disease patients (AD) and control individuals. Levels of Aβ antibodies in CSF from AD patients were reduced by 30% when compared to age-matched control subjects ($p<0.05$, one way ANOVA)

[0037] **FIG. 2**: 1 AD antibody unit=10 antibody titres. 1 ml of CSF was incubated with 1, 10, or 100 μl of protein A conjugated with agarose bead (Sigma P-7786) overnight at 4° C. (Pa). After removing protein A, 290 μl of CSF were used to determine the titre of antibody. 1 ml of CSF was also incubated with $Aβ_{1-40}$, $Aβ_{1-42}$, $Aβ_{25-35}$ (1 mg Aβ was dissolved in 0.9 ml of deionised H_2O). 0.8-20 μL of Aβ was used for overnight incubation (at 4° C.) with CSF. 290 ml of CSF was then used for determination.

[0038] PE 1-100 μL: Protein A precipitates (1, 10, or 100 μl) from CSF sample was incubated with 100 μl of PBS (pH 2.5). The recovered solution was used for titre determination. The antibody titre is defined as the dilution of antibody that gives a half-maximal binding to antigen. (Pa: Protein A; $Aβ_{1-40}$: β-Amyloid **1-40**; $Aβ_{1-42}$: β-Amyloid 1-42; $Aβ_{25-35}$: β-Amyloid 25-35; PE: elute from protein A precipitates)

[0039] **FIG. 3**: Same condition as in **FIG. 2**. Only $Aβ_{1-40}$=2 μl. $Aβ_{40-1}$=2 μl. Neuropeptide F and neuropeptideY (2 μl), Amylin (2 μl).

[0040] **FIG. 4**: Purification of anti-Aβ antibodies by using Aβ affinity column. After 250 g immunoglobulin (IgG) pass through the Aβ affinity column, 10 ml of elute buffer (pH2.5) was used to elute Aβ antibody. Then another 10 ml of elute buffer (pH1.5) was used to elute the remainder of antibodies. After ELISA detection, significant amount of Aβ antibody was detected in pH 2.5 elute buffer. IgG=immunoglobulin 100 μl. PH2.5, 1.0: elute antibodies by using pH2.5 and then pH1.5 buffers from affinity column: 100 μl. PT: IgG pass through Aβ affinity column, equal to 100 μl of IgG. Most anti-Aβ antibody elute from column by pH 2.5. Column: 3 mg of $Aβ_{1-40}$ was conjugated into Sepharose 4B (Pharmacia, 5 ml). Purification by using Pharmacia FPLC system at 4 degree. 1 Aβ antibody unit=10 antibody titres. (Elute buffer: 50 mM glycine, 150 mM NaCl, pH 2.5).

[0041] **FIG. 5**: Concentration of β-Amyloid in the CSF before treatment with immunoglobulins and 7-12 days and 4 weeks after treatment, respectively. Measurements were done as described in Example 2.

[0042] The following examples are given for illustration purposes only and are not intended to limit the scope of the invention.

EXAMPLE 1

Treatment of AD Patients by Infusion of Human IgG Immunoglobulins or Anti-Aβ Antibodies from Human IgG

[0043] As an example as to the therapy regimen 5-30 g (1-5 days) of IgG immunoglobulins (commercially available) or a corresponding amount of purified anti-Aβ antibody are administered parenterally to the patient by the i.v. route. Levels of β-Amyloid, tau-protein as well as Aβ-antibody are measured in the serum and CSF before and following the respective dose of IgG immunoglobulins for therapy control. The goal is to decrease β-amyloid concentration in the CSF and by that decrease the plaque burden in Alzheimer's disease and alleviate the neuropsychiatric and neuropsychological defects in Alzheimer's disease. This treatment introduces a new therapeutic approach to Alzheimer's disease.

EXAMPLE 2

Effect of i.v. Immunoglobulins on the Concentration of β-Amyloid in the CSF

[0044] In this example the effect of the application of i.v. immunoglobulins (Octagam®, Polyglobulin®) on the concentration of β-Amyloid in the CSF is investigated.

[0045] Four patients suffering from different neurological disorders (Guillain-Barre-Syndrome; chronic inflammatory demyelinating neuropathy, CIDP) were included in this study. Lumbar CSF was withdrawn before starting treatment with i.v. immunoglobulins. After 7 to 12 days and 4 weeks an additional lumbar puncture was performed. The withdrawal of CSF was performed during regular investigations. Patients were treated with i.v. immunoglobulins for 3-5 days with 0.4 g/kg per day before withdrawal of CSF. The concentration of β-Amyloid was measured in the CSF before treatment and 7-12 days and 4 weeks after application of i.v. immunoglobulins. The results are shown in **FIG. 5**.

[0046] From the figure it can be seen that the amount of β-Amyloid was reduced from 1835 ng/l before treatment to 1622 ng/l(7-12 d after treatment) and 1376 ng/l (4 weeks after treatment). These results show that i.v. administration of immunoglobulins has an effect on the concentration of β-Amyloid in the CSF. Immunoglobulins also reduce β-Amyloid in the brain of patients with Alzheimer's disease.

1. A human anti-Aβ-amyloid antibody obtained by purification from a human IgG-containing bodyfluid by Aβ-affinity chromatography.

2. Human anti-Aβ-amyloid antibody of claim 1, characterized in that it belongs to the class of immunoglobulines G (IgG) and does not recognize $Aβ_{40-1}$, neuropeptide F, neuropeptide Y, and Amylin, and specifically recognizes one or more of $Aβ_{1-40}$,$Aβ_{1-42}$ $Aβ_{25-35}$.

3. A method of purification of an anti-Aβ-amyloid antibody comprising the steps of

(i) obtaining a human IgG-containing bodyfluid,

(ii) subjecting the bodyfluid obtained to an Aβ-affinity chromatography, and

(iii) recovering the purified anti-Aβ antibody.

4. The method of claim 3 wherein the IgG-containing bodyfluid is a cerebrospinal fluid, plasma or urine obtained from one or more human beings (pooled samples).

5. The method of claim 3, wherein A-affinity chromatography is carried out by an Aβ-affinity column, obtained by conjugating $Aβ_{1-40}$ onto Sepharose 4B, elution with elute buffer at pH 1.5 to 2.5 at 4° C. using an FPLC system.

6. Use of an anti-Aβ-amyloid antibody according to claims **1** or **2** for treating amyloid associated diseases, especially Alzheimer's disease and primary and secondary amyloidoses.

7. Use of an anti-Aβ-amyloid antibody according to claims **1** or **2** for diagnosis of amyloid associated diseases, especially Alzheimer's disease and primary and secondary amyloidoses.

8. Use of an IgG containing, preferably IgG enriched fluid for treatment of amyloid associated diseases, especially Alzheimer's disease.

9. Pharmaceutical composition comprising an anti-Aβ-amyloid antibody according to claims **1** or **2**.

* * * * *

PCT

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International Bureau

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(54) Title: METHODS FOR PRODUCING PURE PERLECAN AND OTHER HEPARAN SULFATE PROTEOGLYCANS

(57) Abstract

A method of perlecan isolation (from the EHS tumor) which produces "clean" (i.e., substantially "pure") perlecan is disclosed. Clean perlecan is thus produced in sufficient quantities for use in a number of different *in vitro* and *in vivo* assays. In addition, this isolation method exploits a newly discovered aggregating property of a ~220 kDa heparan sulfate proteoglycan (HSPG) observed during gel filtration chromatography, which allows it to be effectively separated from non-aggregating perlecan. The method employs specific cation exchange, anion exchange, molecular sieve chromatography and immobilized GAG affinity chromatography. It is demonstrated that there are no other contaminating proteins in the perlecan and HSPG preparations, and that the perlecan core protein is intact. Improved, clean perlecan based, rodent models of fibrillar amyloid protein deposition, accumulation and/or persistence in tissues are disclosed.

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Title: METHODS FOR PRODUCING PURE PERLECAN AND OTHER HEPARAN SULFATE PROTEOGLYCANS

TECHNICAL FIELD

The invention relates to methods for producing pure perlecan and other heparan sulfate proteoglycans; more particularly, it relates to methods for isolating and purifying perlecan and other heparan sulfate proteoglycans from tissue extracts by means of column chromatography; moreover, it relates to the pure perlecan and other heparan sulfate proteoglycans produced by these methods and to assays and animal models employing these pure substances.

BACKGROUND OF THE INVENTION

Perlecan is a specific heparan sulfate proteoglycan (HSPG) and a common constituent of all amyloid deposits regardless of the specific amyloid protein involved. Perlecan is believed to play primary roles in the pathogenesis of amyloidosis and contributes to the formation, deposition, accumulation and/or persistence of amyloid in a variety of tissues and in different clinical settings.

However, perlecan is an extremely difficult macromolecule to isolate in pure form, especially in substantial quantities, due in part to perlecan's inherent ability to interact with a number of different proteins and macromolecules. The most commonly utilized extract source for isolation of perlecan is the Engelbreth-Holm-Swarm (EHS) tumor which is routinely grown in the hind legs of mice. However, major problems still exist with trying to obtain "clean" perlecan from this basement membrane producing tumor, especially when using known methods of isolation. These problems include: a) contamination by other proteins and/or basement membrane components of the EHS tumor including laminin, fibronectin and type IV collagen which all tend to interact with perlecan, b) contamination due to the presence of free glycosaminoglycan (GAG) chains, and c) degradation of the perlecan core protein.

Thus, there is a need in the art to develop effective new ways to isolate perlecan in high yield and of high quality (*i.e.* "clean" or substantially "pure" perlecan) in order to use this product for a number of important biological assays which have impact on a number of major human diseases including Alzheimer's disease, Down's syndrome, type II diabetes, certain forms of cancer and inflammatory disorders. All of these latter diseases involve amyloid accumulation and persistence, and involve perlecan and related HSPGs in the pathogenesis. Isolation of perlecan for use in further understanding perlecan's role in the pathogenesis of these major human disorders and for the eventual identification of new therapeutics will be of tremendous benefit.

DISCLOSURE OF THE INVENTION

Summary of the Invention

The present invention describes methods of perlecan isolation and purification which produce "clean" perlecan, and do so in sufficient quantities for effective use in a number of different *in vitro* and *in vivo* assays. In addition, this isolation method does not require cesium chloride (or other density gradient) centrifugation and exploits a newly discovered aggregating property of a ~220 kilodalton (kDa) heparan sulfate proteoglycan (HSPG) observed during gel filtration (*i.e.* molecular sieve) chromatography, which allows it to also be effectively isolated separately from non-aggregating perlecan.

In these methods, 50-200 grams of EHS tumor are routinely extracted using 4M guanidine-HCl, followed by specific cation exchange, anion exchange and/or molecular sieve chromatography. SDS-PAGE analysis (before and after digestion with heparitinase/ heparinase or nitrous acid) followed by staining with silver, demonstrates no other contaminating proteins in the perlecan preparations. Western blots using a specific perlecan core protein antibody (HK-102) following heparitinase digestion show a characteristic doublet at 400 and 360 kDa indicative of intact perlecan core protein. Absence of contamination by other basement membrane components produced by the EHS tumor is confirmed by absence of immunoreactive bands on Western blots using antibodies against laminin, fibronectin or type IV collagen.

The present invention describes detailed isolation and characterization of perlecan and a ~220 kDa HSPG from the EHS tumor to ensure quantity production of perlecan and the ~220 kDa HSPG of the highest quality, and to maximize the potential effects of these products using *in vitro* assays, and in a rodent model of fibrillar beta-amyloid protein deposition, accumulation and/or persistence in brain tissue. In

addition, the methodology describes the unique isolation of a ~220 kDa HSPG from the EHS tumor. Lastly, several aspects of the isolation methodology described, including the use of anionic resins (*i.e.* cationic exchange resins) and immobilized glycosaminoglycans (GAGs), can also be applied, as for instance following conventional extraction techniques, to obtain preparations of other "clean" proteoglycans (PGs) from different tissue, organ, tumor or cell culture sources.

Features of the Invention

Perlecan is a specific HSPG which is present on all basement membranes (Dziadek et al., EMBO J. 4:905-912, 1985; Kato et al., J. Cell Biol. 106:2203-2210, 1988; Murdoch et al., J. Histochem. Cytochem. 42:239-249, 1988). Perlecan is believed to play fundamental roles in the pathogenesis of Alzheimer's disease (AD) amyloidosis, as well as in other types of central nervous system and systemic amyloidoses (reviewed in Snow and Wight, Neurobiol. Aging 10:481-497, 1989). Perlecan can be routinely isolated from the EHS mouse tumor. However, it is extremely difficult to isolate perlecan in "pure" form and in sufficient quantities due to perlecan's inherent ability to interact with other basement membrane components (*i.e.* laminin, fibronectin and/or type IV collagen) which are also produced by the EHS tumor. This invention relates to detailed methodology that allows one to isolate perlecan in relatively "pure" or "clean" form and in sufficient quantities for use in a number of different and relevant *in vitro* and *in vivo* assays. Some of the work disclosed here has been reported by GM Castillo, JA Cummings, C Ngo, W Yang and AD Snow in a manuscript entitled Novel Purification and Detailed Characterization of Perlecan Isolated from the Engelbreth-Holm-Swarm Tumor for Use in an Animal Model of Fibrillar Aß Amyloid Persistence in Brain, in J. Biochem. 120:433-444, 1996, the substance of which is hereby incorporated by reference.

A primary object of the present invention is to provide methods for the isolation of intact and "clean" (*i.e.* substantially "pure") perlecan which does not contain contaminating proteins or other macromolecules. The terms "clean" "substantially pure" and/or "free (of contaminants)" are used herein to refer to isolated perlecan or HSPGs that contain less than 1% (and preferably 0.1% or less) by weight of contaminating proteins, other macromolecules or DNA.

Yet another object of the present invention is to provide methods for the isolation of a quantity of intact and "clean" HSPG such as perlecan which preferably contains substantially no (less than 1% and preferably less than or equal to 0.1%)

contaminating basement membrane components including laminin, fibronectin, or type IV collagen.

Yet another object of the present invention is to provide methods for the isolation of a quantity of intact and "clean" HSPG such as perlecan which preferably contains substantially no (less than 1% and preferably less than or equal to 0.1%) of free glycosaminoglycan (GAG) chains.

Yet another object of the present invention is to provide methods for the isolation of a quantity of intact and "clean" HSPG such as perlecan which preferably contains substantially no (less than 1% and preferably less than or equal to 0.1%) of potential contaminating DNA.

Yet another object of the present invention is to provide methods for the consistent production of "clean" perlecan in sufficient quantities for use in a number of different and relevant *in vitro* and *in vivo* biological assays.

Yet another object of the present invention is to provide like methods to isolate other proteoglycans from human and animal tissues or cell culture, such that the isolated PGs are essentially "free" of contaminating laminin, fibronectin, type IV collagen and/or other matrix proteins (*i.e.* those proteins that normally interact with proteoglycans).

Yet another object of the present invention is to provide methods to remove PG-associated proteins or macromolecules in order to obtain "clean" or substantially "pure" PG preparations.

Yet another object of the present invention is to provide methods for the use of immobilized GAGs to remove PG-associated proteins or macromolecules in order to obtain "clean" or substantially "pure" PG preparations.

Yet another object of the present invention is to provide methods for the use of cation exchange resins to remove PG-associated proteins or macromolecules in order to obtain "clean" or substantially "pure" PG preparations.

Another aspect of the present invention is to use "clean" perlecan and/or ~220 kDa HSPG (*i.e.* perlecan and/or ~220 kDa HSPG produced by one or more methods of the invention) to establish new therapeutic methods and diagnostic applications for the amyloid diseases, methods otherwise impractical with conventionally prepared perlecan or HSPG.

The amyloid diseases referred to herein include, but are not limited to, the amyloid associated with Alzheimer's disease and Down's syndrome (wherein the specific amyloid is referred to as beta-amyloid protein or Aß), the amyloid associated

with chronic inflammation, various forms of malignancy and Familial Mediterranean Fever (wherein the specific amyloid is referred to as AA amyloid or inflammation-associated amyloidosis), the amyloid associated with multiple myeloma and other B-cell dyscrasias (wherein the specific amyloid is referred to as AL amyloid), the amyloid associated with type II diabetes (wherein the specific amyloid is referred to as amylin or islet amyloid), the amyloid associated with the prion diseases including Creutzfeldt-Jakob disease, Gerstmann-Straussler syndrome, kuru and animal scrapie (wherein the specific amyloid is referred to as PrP amyloid), the amyloid associated with long-term hemodialysis and carpal tunnel syndrome (wherein the specific amyloid is referred to as beta$_2$-microglobulin amyloid), the amyloid associated with senile cardiac amyloid and Familial Amyloidotic Polyneuropathy (wherein the specific amyloid is referred to as transthyretin or prealbumin), and the amyloid associated with endocrine tumors such as medullary carcinoma of the thyroid (wherein the specific amyloid is referred to as variants of procalcitonin).

Another aspect of the present invention is to use "clean" perlecan and/or ~220 kDa HSPG to produce new polyclonal and/or monoclonal antibodies of a previously unattainable purity and integrity which can then be employed in a number of *in vitro* assays to specifically detect perlecan, perlecan derived-fragments, the ~220 kDa HSPG and ~220 kDa HSPG-derived fragments in human tissues and/or biological fluids. Polyclonal or monoclonal antibodies made specifically against "clean" perlecan, a protein core fragment of "clean" perlecan (which interacts with specific amyloid proteins), or the ~220 kDa HSPG can be put to a most effective use to detect and quantify amyloid disease specific perlecan or ~220 kDa HSPG fragments in human tissues and/or biological fluids, and to a previously unattainable degree of precision. These antibodies can be made by administering the "clean" perlecan or the ~220 kDa HSPG in antigenic form to a suitable host. Polyclonal or monoclonal antibodies may be prepared by standard techniques known to those skilled in the art.

Another object of the present invention is to use perlecan or ~220 kDa HSPG-specific antibodies referred to above for the detection and specific localization of perlecan, the ~220 kDa HSPG or their fragments in human tissues, cells, and/or cell culture using standard immunohistochemical techniques, known to those skilled in the art.

Yet another aspect of the present invention is to use antibodies recognizing perlecan, ~220 kDa HSPG, or fragments thereof, for *in vivo* labeling; for example, with

a radionucleotide, for radioimaging to be utilized for *in vivo* diagnosis, and/or for *in vitro* diagnosis.

Another object of the present invention is to use clean perlecan and/or perlecan-derived peptides or fragments for use in *in vitro* assays to detect perlecan autoantibodies in human biological fluids. Specific assay systems can be utilized to not only detect the presence of perlecan autoantibodies in biological fluids, but also to monitor the progression of disease by following elevation or diminution of perlecan autoantibody levels.

Another aspect of the invention is to use clean perlecan, perlecan fragments, perlecan-derived peptides, ~220 kDa HSPG or fragments thereof, to generate antibodies and/or molecular biology probes for the detection of perlecan or the ~220 kDa HSPG in human tissues in the amyloid diseases.

Yet another aspect of the present invention is to utilize "clean" perlecan or ~220 kDa HSPG products for the establishment of new animal models for the deposition, accumulation and/or persistence of fibrillar Aß amyloid in brain as observed in Alzheimer's disease and Down's syndrome. These new animal models can be used to effectively screen and identify new therapeutic agents that target fibrillar Aß amyloid formation, deposition, accumulation and/or persistence in brain.

Yet another aspect of the present invention is to provide new animal models for the production, deposition, accumulation and/or persistence of fibrillar amyloid as observed in each of the other amyloidoses, by employing the "clean" perlecan of the invention. This includes, but is not limited to, the amyloid associated with chronic inflammation, various forms of malignancy and Familial Mediterranean Fever (wherein the specific amyloid is referred to as AA amyloid or inflammation-associated amyloidosis), the amyloid associated with multiple myeloma and other B-cell dyscrasias (wherein the specific amyloid is referred to as AL amyloid), the amyloid associated with type II diabetes (wherein the specific amyloid is referred to as amylin or islet amyloid), the amyloid associated with the prion diseases including Creutzfeldt-Jakob disease, Gerstmann-Straussler syndrome, kuru and animal scrapie (wherein the specific amyloid is referred to as PrP amyloid), the amyloid associated with long-term hemodialysis and carpal tunnel syndrome (wherein the specific amyloid is referred to as $beta_2$-microglobulin amyloid), the amyloid associated with senile cardiac amyloid and Familial Amyloidotic Polyneuropathy (wherein the specific amyloid is referred to as transthyretin or prealbumin), and the amyloid associated with endocrine tumors such as medullary carcinoma of the thyroid (wherein the specific amyloid is referred

to as variants of procalcitonin). These new animal models can also be used for the evaluation of candidate drugs and therapies for the prevention and treatment of the amyloidoses as referred to above.

In a particular aspect of the invention there is a method of preparation of substantially pure proteoglycan from an extract source, with the method using some or all of the following steps: a) isolation of an extracted proteoglycan by molecular sieve column chromatography, b) cation exchange column chromatography, c) anion exchange column chromatography, and d) chromatography using a column containing immobilized glycosaminoglycans. The proteoglycan referred to is advantageously perlecan, but can be the ~220 kDa HSPG (aggregating or not), or fragments thereof, and the preferred though not exclusive extract source is Engelbreth-Holm-Swarm tumor tissue.

The isolation by molecular sieve column chromatography employs a Sephacryl S-400 column; the isolation by cation exchange column chromatography employs a Sulphopropyl Sepharose column; the isolation by anion exchange column chromatography employs a DEAE-Sephacel column; the isolation by a molecular sieve column employs a second column in the form of a Sephacryl S-1000 column; the isolation by a column containing immobilized glycosaminoglycans employs a heparin-Sepharose column. In each variation of the method, isolation proceeds to a level of contaminating proteins, proteoglycans or macromolecules of less than 1%, or to a level of contamination by DNA of less than 1%, and preferably proceeds in any case to a level of contaminating proteins, proteoglycans or macromolecules of less than or equal to 0.1%.

Another aspect of the invention is the clean perlecan produced by the method of the invention, having a level of contaminating proteins, proteoglycans, macromolecules or DNA of less than 1%. Another aspect is a method of making an antibody, with the method producing antibodies from the clean perlecan. The making of the antibody includes the step of production of at least one type of antibody selected from the group of antibody types consisting of polyclonal, monoclonal, and chimeric antibodies and anti-idiotypic antibodies.

There is also a disclosed a method to diagnose a disease or a susceptibility to a disease related to the levels of perlecan, perlecan-derived protein or glycosaminoglycans fragments, ~220 kDa HSPG, or ~220 kDa HSPG-derived protein or glycosaminoglycans fragments, the method comprising determining levels of perlecan, a particular perlecan-derived fragment, or the ~220 kDa HSPG, or ~220 kDa derived fragment in

a sample, whereby the levels are indicative of the presence of a disease, susceptibility to a disease, or progression of said disease, such as an amyloid disease. This method can advantageously include the step of radiolabeling the antibodies for radioimaging or *in vivo* diagnosis for detection of perlecan, perlecan-derived protein or glycosaminoglycans fragments, ~220 kDa HSPG, or ~220 kDa HSPG-derived protein or glycosaminoglycan fragments.

There is also disclosed a method for detection and quantification of perlecan and perlecan-derived fragments in biological fluids comprising a) allowing a first clean perlecan or perlecan-derived fragment antibody to bind to microtiter wells for a sufficient time to allow said binding, b) adding a quantity of biological fluid to the microtiter wells, c) incubating the biological fluid for sufficient time to allow binding of any perlecan or perlecan-derived fragment in the biological fluid to the first antibody on the microtiter wells, d) adding a second labeled antibody to the microtiter wells wherein the second labeled antibody is against perlecan or perlecan-derived fragment, but which is against a different epitope than the first antibody, and allowing the second antibody to bind to any perlecan or perlecan-derived fragment captured by the first antibody, and e) detecting bound materials using an appropriate substrate or label.

There is also disclosed a method for detection and quantification of perlecan autoantibodies in biological fluids comprising a) allowing clean perlecan or a fragment thereof to bind to microtiter wells for a sufficient time to allow said binding, b) adding a quantity of biological fluid to the microtiter wells, c) incubating the biological fluid for sufficient time to allow binding of any perlecan autoantibody in the biological fluid to the clean perlecan or a fragment thereof on the microtiter wells, d) adding a labeled antibody to the microtiter wells wherein the labeled antibody is against human immunoglobulins and allowing the antibody to bind to any perlecan autoantibody captured by the perlecan or a fragment thereof, and e) detecting bound materials using an appropriate substrate or label.

In any of the methods of the invention, the biological fluids may be blood, plasma, serum, cerebrospinal fluid, sputum, saliva, urine and stool or the like.

There is also disclosed a method for the treatment of a patient having an identified clinical need to interfere with the pathological effects of amyloid, the method comprising administrating to the patient a therapeutically effective amount of clean perlecan, or a protein or glycosaminoglycan fragment thereof, where the amyloid disease is Alzheimer's disease.

There is also disclosed a method for producing an animal model of amyloid diseases comprising a) injecting or infusing clean perlecan, the ~220 kDa HSPG, or fragments thereof, in combination with the appropriate amyloid protein into a given tissue or organ of a non-human mammal, and b) allowing sufficient time for the amyloid protein plus perlecan or the ~220 kDa HSPG to be co-deposited in said tissue or organ, c) detecting the amyloid deposit in said organ in tissue using standard staining techniques for fibrillar amyloid. In this method the amyloid disease may be Alzheimer's disease and the appropriate amyloid protein may be the beta-amyloid protein (Aß). The tissue or organ may be brain tissue, and the non-human mammal may be a rat or other rodent.

There is also disclosed an *in vivo* assay for selecting a candidate therapeutic for inhibiting congophilic and fibrillar amyloid deposition/persistence, comprising a) administering a candidate reagent to a first animal in a first infusate comprising an amyloid protein and clean perlecan or the ~220 kDa HSPG by continuous infusion at an infusion site into said tissue or organ, b) selecting the candidate reagent as a candidate therapeutic for inhibiting congophilic and fibrillar amyloid deposition/persistence if the first infusate diminishes Congo red and Thioflavin S staining indicative of fibrillar amyloid deposition/persistence at the infusion site, as compared with a second animal receiving a second infusate consisting essentially of the amyloid protein and perlecan or the ~220 kDa HSPG.

A method of perlecan isolation (from the EHS tumor) which produces "clean" (*i.e.* substantially "pure") perlecan is disclosed. Clean perlecan is thus produced in sufficient quantities for use in a number of different *in vitro* and *in vivo* assays. In addition, this isolation method exploits a newly discovered aggregating property of a ~220 kDa heparan sulfate proteoglycan (HSPG) observed during gel filtration chromatography, which allows it to be effectively separated from non-aggregating perlecan. The method employs specific cation exchange, anion exchange, molecular sieve chromatography and immobilized GAG affinity chromatography. It is demonstrated that there are no other contaminating proteins in the perlecan and HSPG preparations, and that the perlecan core protein is intact. Improved, clean perlecan based, rodent models of fibrillar amyloid protein deposition, accumulation and/or persistence in tissues are disclosed.

These and other features and advantages of the present invention will become more fully apparent when the following detailed description of the invention is read in conjunction with the accompanying figures.

BRIEF DESCRIPTION OF THE DRAWINGS

The following drawings are illustrative of embodiments of the invention and are not meant to limit the scope of the invention.

FIGURE 1 is a schematic describing a protocol for clean perlecan purification (isolation) from the Engelbreth-Holm-Swarm tumor.

FIGURE 2 is a color photograph of a SDS-PAGE gel stained with Alcian blue followed by Coomassie blue, which represents an analysis of the purity of perlecan, other proteoglycans, and/or proteins, at each step of the isolation protocol.

FIGURE 3 is a black and white photograph of an Alcian blue stained SDS-PAGE gel of fractions obtained from a third pass through a Sephacryl S-1000 column.

FIGURE 4 is a black and white photograph of silver and Alcian blue stained SDS-PAGE gels to assess the purity of the final perlecan preparations.

FIGURE 5 is a black and white photograph of silver staining of the final perlecan preparations to ensure intact perlecan core protein and absence of any contaminating proteins/ proteoglycans.

FIGURE 6 is a black and white photograph of Western blot analysis to demonstrate the absence of laminin, fibronectin or type IV collagen in the final perlecan preparations.

FIGURE 7 is a schematic describing another protocol employed for perlecan purification from the Engelbreth-Holm-Swarm tumor with further modification steps to improve both clean perlecan yield, and the time required for isolation.

FIGURE 8 is a color photomicrograph demonstrating the infusion of the perlecan product plus beta-amyloid protein (Aß) into rodent hippocampus for the establishment of a reliable animal model of Aß amyloid deposition/ persistence.

BEST MODE OF CARRYING OUT THE INVENTION

The following sections are provided by way of additional background to better appreciate the invention.

Structure of Perlecan

The DNA sequence for human perlecan encodes a protein core with a molecular weight of approximately 467 kDa (Murdoch, A.D. et al, J. Biol. Chem. 267:8544-8557, 1992) whereas the DNA sequence for mouse perlecan encodes a protein core with a molecular weight of approximately 396 kDa (Noonan, D.M. et al, J. Biol. Chem.

266:22939-22947, 1991). The genes for human (Murdoch, A.D. et al, J. Biol. Chem. 267:8544-8557, 1992; Kallunki, P. and Tryggvason, K. J. Cell Biol. 116:559-571, 1992) and mouse (Noonan, D.M. et al, J. Biol. Chem. 266:22939-22947, 1991) perlecan have been cloned and the predicted core protein consists of five distinct domains. Domain I contains the proposed heparan sulfate GAG attachment sites and is unique to perlecan showing no similarity to other known protein sequences. The location of the three Ser-Gly consensus heparan sulfate GAG attachment sites at the N-terminus corresponds with the number and position of known GAG chains (Kokenyesi, R. and Silbert, J.E. Biochem. Biophys. Res. Comm. 211: 262-267, 1995). Domain II is homologous to the LDL binding domain present in the LDL-receptor, whereas Domain III has homology to the globule-rod regions of the laminin short arms. Domain IV is a highly repetitive region with numerous immunoglobulin-like repeats that show the highest similarity to neural cell adhesion molecule (N-CAM). Domain V has three globular repeats very similar to the domain G repeats in the laminin A chain and the equivalent segment of the A chain homologue, merosin, and two epidermal growth factor-like regions (Noonan, D.M., and Hassell, J.R., Kidney Int. 43:53-60, 1993). The perlecan core protein is therefore a unique and large macromolecule with homology to a number of other well known proteins.

Perlecan Production by Different Cell Types and its Postulated Roles in the Pathogenesis of Amyloidoses

Perlecan is present on all basement membranes (Dziadek et al, EMBO J. 4, 905-912, 1985; Kato et al, J. Cell Biol. 106:2203-2210, 1988; Murdoch et al, J. Histochem. Cytochem. 42: 239-249, 1994) and was previously cloned from both human (Murdoch et al, J. Biol. Chem. 267: 8544-8557, 1992; Kallunki and Tryggvason, J. Cell. Biol. 116:559-571, 1992) and mouse (Noonan et al, J. Biol. Chem. 266:22939-22947, 1991). Perlecan is known to be produced by different cell types including endothelial cells (Kinsella and Wight, Biochem. 27:2136-2144, 1988; Saku and Furthmayr, J. Biol. Chem. 264:3514-3523, 1989; Rescan et al, Am. J. Path. 142:199-208, 1993), smooth muscle cells (Nikkari et al, Am. J. Path. 144: 1348-1356, 1994), fibroblasts (Murdoch et al, J. Histochem. Cytochem. 42:239-249, 1994; Heremans et al, J. Cell Biol. 109:3199-3211, 1989), epithelial cells (Morris et al, In Vitro Cell Dev. Biol. 30:120-128, 1994; Ohji et al, Invest. Opth. Vis. Sci. 35:479-485, 1994; Van Det et al, Biochem. J. 307:759-768, 1995), and synovial cells (Dodge et al, Lab. Invest. 73:649-657, 1995). Perlecan is also synthesized by bone marrow derived cells (Grässel et al, Mol. Cell Biochem. 145:61-68, 1995) and is present in cancerous tissue including metastatic

melanomas (Cohen et al, Cancer Res. 54:5771-5774, 1994), human breast tumors (Guelstein et al, Int. J. Cancer 53:269-277, 1993), and liver tumors (Kovalsky et al, Acta Biomed. Ateneo Parmense 64:157-163, 1993). Both F9 embryonal carcinoma cells (which form parietal endoderm) and P19 embryonal carcinoma cells (which form cholinergic neurons) also demonstrate marked increased perlecan expression and synthesis upon differentiation (Chakravarti et al, Dev. Dyn. 197:107-114, 1993; Sekiguchi et al, J. Neurosc. Res. 38:670-686, 1994).

Perlecan is postulated to play a primary role in the pathogenesis of Alzheimer's disease (AD) amyloidosis, as well as in other types of central nervous system and systemic amyloidoses (reviewed in Snow, and Wight, Neurobiol. Aging 10:481-497, 1989). Only heparan sulfate proteoglycans have been found to be immunolocalized to all three major lesions (*i.e.* neuritic plaques, neurofibrillary tangles and cerebrovascular amyloid deposits) in Alzheimer's disease brain and specifically to the beta-amyloid protein (Aß)-containing amyloid fibrils in both amyloid plaques and congophilic angiopathy (Snow et al, Am. J. Path. 133:456 -463, 1988; Snow and Wight, Neurobiol. Aging 10:481-497, 1989; Perlmutter and Chui, Brain Res. Bull. 24:677-686, 1990; Snow et al, Am. J. Path. 137:1253-1270, 1990; Su et al, Neuroscience 51:801-813,1992; Van Gool et al, Dementia 4:308-314, 1993). Accumulating evidence suggests that perlecan is a major heparan sulfate proteoglycan present within the Aß-containing amyloid deposits in Alzheimer's disease (Snow et al, Am. J. Path. 133:456 -463, 1988; Snow and Wight, Neurobiol. Aging, 10:481-497, 1989; Snow et al, Am. J. Path. 137:1253-1270, 1990; Snow et al, Am. J. Path. 144:337-347, 1994) and may play a primary role in Aß fibril formation, deposition, accumulation and persistence. The consistent co-localization of perlecan to Aß deposits which exist in both a fibrillar and non-fibrillar form (Snow et al, Am. J. Path. 144:337-347, 1994) is probably due to perlecan's high affinity interactions with Aß (Snow et al, J. Neuropath. Exp. Neurol. 48:352, 1989 Abstract; Buee et al, Brain Res. 601:154-163, 1993; Buee et al, Brain Res. 627:199-204, 1993; Snow et al, Arch. Biochem. Biophys. 320:84-95, 1995) and with beta-amyloid precursor proteins (Narindrasorasak et al, J. Biol. Chem. 266:12878-12883, 1991). Residues 13-16 of Aß have been identified as a perlecan binding site (Snow et al, J. Neuropath. Exp. Neurol. 48:352, 1989 Abstract; Brunden et al, J. Neurochem. 61:2147-2154, 1993; Snow et al, Arch. Biochem. Biophys. 320:84-95, 1995). This region contains a heparin/heparan sulfate binding consensus sequence (Cardin and Weintraub, Arterioscl. 9:21-32, 1989), and is adjacent to the postulated alpha-secretase cleavage site on Aß (at Lys-16). Once bound, perlecan is

believed to influence the secondary structure and/or aggregation properties of Aß and/or beta-amyloid precursor proteins (Fraser et al, J. Neurochem. 59:1531-1540, 1992). Perlecan also appears to play a role in stabilizing fibrillar Aß amyloid when deposited *in vivo* (Snow et al, Neuron 12:219-234, 1994; Snow et al, Soc. Neurosc. Abst. 21:1292, 1995 Abstract), and protects Aß from degradation by proteases as recently demonstrated *in vitro* (Gupta-Bansal et al, J. Biol. Chem. 270:18666-18671, 1995). The combined results described above suggest that perlecan is an important macromolecule that has now been implicated at several key steps in the pathogenesis of Aß amyloidosis in AD.

Other Amyloid Diseases

The "amyloid diseases" consist of a group of clinically and generally unrelated human diseases which all demonstrate a marked accumulation in tissues of an insoluble extracellular substance known as "amyloid", and usually in an amount sufficient to impair normal organ function. Rokitansky in 1842 (Rokitansky, "Handbuch der pathologischen Anatomie", Vol. 3, Braumuller and Seidel, Vienna) was the first to observe waxy and amorphous looking tissue deposits in a number of tissues from different patients. However, it wasn't until 1854 when Virchow (Virchow, Arch. Path. Anat. 8:416, 1854) termed these deposits as "amyloid" meaning "starch-like" since they gave a positive staining with the sulfuric acid-iodine reaction, which was used in the 1850's for demonstrating cellulose. Although cellulose is not a constituent of amyloid, nonetheless, the staining that Virchow observed was probably due to the present of proteoglycans (PGs) which appear to be associated with all types of amyloid deposits. The name amyloid has remained despite the fact that Friederich and Kekule in 1859 discovered the protein nature of amyloid (Friedrich and Kekule, Arch. Path. Anat. Physiol. 16:50, 1859). For many years, based on the fact that all amyloids have the same staining and structural properties, lead to the postulate that a single pathogenetic mechanism was involved in amyloid deposition , and that amyloid deposits were thought to be composed of a single set of constituents. Current research has clearly shown that amyloid is not a uniform deposit and that amyloids may consist of different proteins which are totally unrelated (Glenner, N. England J. Med. 302:1283-1292, 1980).

Although the nature of the amyloid itself has been found to consist of completely different and unrelated proteins, all amyloids appear similar when viewed under the microscope due to amyloid's underlying protein able to adapt into a fibrillar structure. All amyloids regardless of the nature of the underlying protein 1) stain

characteristically with the Congo red dye and display a classic red/green birefringence when viewed under polarized light (Puchtler et al, J. Histochem. Cytochem. 10:355-364, 1962), 2) ultrastructurally consists of fibrils with a diameter of 7-10 nanometers and of indefinite length, 3) adopt a predominant beta-pleated sheet secondary structure. Thus, amyloid fibrils viewed under an electron microscope (30,000 times magnification) from the post-mortem brain of an Alzheimer's disease patient would look nearly identical to the appearance of amyloid present in a biopsied kidney from a rheumatoid arthritic patient. Both these amyloids would demonstrate a similar fibril diameter of 7-10 nanometers.

In the mid to late 1970's amyloid was clinically classified into 4 groups, primary amyloid, secondary amyloid, familial amyloid and isolated amyloid. Primary amyloid, is amyloid appearing de novo, without any preceding disorder. In 25-40% of these cases, primary amyloid was the antecedent of plasma cell dysfunction such as the development of multiple myeloma or other B-cell type malignancies. Here the amyloid appears before rather than after the overt malignancy. Secondary amyloid, appeared as a complication of a previously existing disorder. 10-15% of patients with multiple myeloma eventually develop amyloid (Hanada et al, J. Histochem. Cytochem. 19:1-15, 1971). Patients with rheumatoid arthritis, osteoarthritis, ankylosing spondylitis can develop secondary amyloidosis as with patients with tuberculosis, lung abscesses and osteomyelitis (Benson and Cohen, Arth. Rheum. 22:36-42, 1979; Kamei et al, Acta Path. Jpn. 32:123-133, 1982; McAdam et al, Lancet 2:572-575, 1975). Intravenous drug users who self-administer and who then develop chronic skin abscesses can also develop secondary amyloid (Novick, Mt. Sin. J. Med. 46:163-167, 1979). Secondary amyloid is also seen in patients with specific malignancies such as Hodgkin's disease and renal cell carcinoma (Husby et al, Cancer Res. 42:1600-1603, 1982). Although these were all initially classified as secondary amyloid, once the amyloid proteins were isolated and sequenced many of these turned out to contain different amyloid proteins.

The familial forms of amyloid also showed no uniformity in terms of the peptide responsible for the amyloid fibril deposited. Several geographic populations have now been identified with genetically inherited forms of amyloid. One group is found in Israel and this disorder is called Familial Mediterranean Fever and is characterized by amyloid deposition, along with recurrent inflammation and high fever (Mataxas, Kidney 20:676-685, 1981). Another form of inherited amyloid is Familial Amyloidotic Polyneuropathy, and has been found in Swedish (Skinner and Cohen, Biochem. Biophys. Res. Comm. 99:1326-1332, 1981), Portuguese (Saraiva et al, J. Lab. Clin.

Med. 102:590-603, 1983; J. Clin. Invest. 74:104-119, 1984) and Japanese (Tawara et al, J. Lab. Clin. Med. 98:811-822, 1981) nationalities. Amyloid deposition in this disease occurs predominantly in the peripheral and autonomic nerves. Hereditary amyloid angiopathy of Icelandic origin is an autosomal dominant form of amyloid deposition primarily affecting the vessels in the brain, and has been identified in a group of families found in Western Iceland (Jennson et al, Clin. Genet. 36:368-377, 1989). These patients clinically have massive cerebral hemorrhages in early life which usually causes death before the age of 40.

The primary, secondary and familial forms of amyloid described above tend to involve many organs of the body including heart, kidney, liver, spleen, gastrointestinal tract, skin, pancreas, and adrenal glands. These amyloid diseases are also referred to as "systemic amyloids" since so many organs within the body demonstrate amyloid accumulation. For most of these amyloidoses, there is no apparent cure or effective treatment and the consequences of amyloid deposition can be detrimental to the patient. For example, amyloid deposition in kidney may lead to renal failure, whereas amyloid deposition in heart may lead to heart failure. For these patients, amyloid accumulation in systemic organs leads to eventual death generally within 3 to 5 years.

Isolated forms of amyloid, on the other hand, tend to involve a single organ system. Isolated amyloid deposits have been found in the lung, and heart (Wright et al, Lab. Invest. 30:767-773, 1974; Pitkanen et al, Am. J. Path. 117:391-399, 1984). Up to 90% of type II diabetic patients (non-insulin dependent form of diabetes) have isolated amyloid deposits in the pancreas restricted to the beta cells in the islets of Langerhans (Johnson et al, New Engl. J. Med. 321:513-518, 1989; Lab. Invest. 66:522-535, 1992). Isolated forms of amyloid have also been found in endocrine tumors which secrete polypeptide hormones such as in medullary carcinoma of the thyroid (Butler and Khan, Arch. Path. Lab. Med. 110:647-649, 1986; Berger et al, Virch. Arch. A Path. Anat. Hist. 412:543-551, 1988). A serious complication of long term hemodialysis is amyloid deposited in the medial nerve and clinically associated with carpal tunnel syndrome (Gejyo et al, Biochem. Biophys. Res. Comm. 129:701-706, 1985; Kidney Int. 30:385-390, 1986). By far, the most common type and clinically relevant type of organ-specific amyloid, and amyloid in general, is that found in the brains of patients with Alzheimer's disease (see U.S. Patent No. 4,666,829 and Glenner and Wong, Biochem. Biophys. Res. Comm. 120:885-890, 1984; Masters et al, Proc. Natl. Acad. Sci. USA 82:4245-4249, 1985). In this disorder, amyloid is predominantly restricted to the central nervous system. Similar deposition of amyloid in the brain

occurs in Down's syndrome patients once they reach the age of 35 years (Rumble et al, New England J. Med. 320:1446-1452, 1989; Mann et al, Neurobiol. Aging 10:397-399, 1989). Other types of central nervous system amyloid deposition include rare but highly infectious disorders known as the prion diseases which include Creutzfeldt-Jakob disease, Gerstmann-Straussler syndrome, and kuru (Gajdusek et al, Science 197:943-960, 1977; Prusiner et al, Cell 38:127-134, 1984; Prusiner, Scientific American 251:50-59, 1984; Prusiner et al, Micr. Sc. 2:33-39, 1985; Tateishi et al, Ann. Neurol. 24:35-40, 1988).

It was misleading to group the various amyloidotic disorders strictly on the basis of their clinical features, since when the major proteins involved were isolated and sequenced, they turned out to be different. For example, amyloid seen in rheumatoid arthritis and osteoarthritis, now known as AA amyloid, was the same amyloid protein identified in patients with the familial form of amyloid known as Familial Mediterranean Fever. Not to confuse the issue, it was decided that the best classification of amyloid should be according to the major protein found, once it was isolated, sequenced and identified.

Thus, amyloid today is classified according to the specific amyloid protein deposited. The amyloid diseases include, but are not limited to, the amyloid associated with Alzheimer's disease, Down's syndrome and hereditary cerebral hemorrhage with amyloidosis of the Dutch type (wherein the specific amyloid is now known as the beta-amyloid protein or Aß), the amyloid associated with chronic inflammation, various forms of malignancy and Familial Mediterranean Fever (AA amyloid or inflammation-associated amyloidosis), the amyloid associated with multiple myeloma and other B-cell abnormalities (AL amyloid), the amyloid associated with type II diabetes (amylin or islet amyloid), the amyloid associated with the prion diseases including Creutzfeldt-Jakob disease, Gerstmann-Straussler syndrome, kuru and animal scrapie (PrP amyloid), the amyloid associated with long-term hemodialysis and carpal tunnel syndrome ($beta_2$-microglobulin amyloid), the amyloid associated with senile cardiac amyloid and Familial Amyloidotic Polyneuropathy (prealbumin or transthyretin amyloid), and the amyloid associated with endocrine tumors such as medullary carcinoma of the thyroid (variants of procalcitonin).

Perlecan Isolation from the Engelbreth-Holm-Swarm Tumor

The most commonly used source for isolation of perlecan is the EHS tumor which is routinely grown in the hind legs of mice (Hassell et al., J. Biol. Chem. 260:8098-8105, 1985; Kato et al., J. Biol. Chem. 262:7180-7188, 1987; Ledbetter et al.,

Biochem. 26:988-995, 1987; Paulsson et al., J. Mol. Biol. 197:297-313, 1987). Known methods of perlecan isolation from the EHS tumor employ a series of extractions and cesium chloride centrifugation (Fujiwara et al., Eur. J. Biochem. 143:145-157, 1984; Hassell et al., J. Biol. Chem. 260:8098-8105, 1985; Kato et al., J. Biol. Chem. 262:7180-7188, 1987; Ledbetter et al., Biochem. 26:988-995, 1987; Paulsson et al., J. Mol. Biol. 197:297-313, 1987). Cesium chloride density gradient centrifugation is employed to separate the two major PGs produced by the EHS tumor, namely perlecan (referred to in the literature as the "low density HSPG"), and a smaller HSPG of M_r 200-400 kDa which has not been fully characterized (referred to as the "high density HSPG") (Fujiwara et al., Eur. J. Biochem. 143:145-157, 1984; Hassell et al., J. Biol. Chem. 260:8098-8105, 1985; Kato et al., J. Biol. Chem. 262:7180-7188, 1987; Ledbetter et al., Biochem. 26:988-995, 1987; Paulsson et al., J. Mol. Biol. 197:297-313, 1987). Some of the shortcomings of using these known methods of perlecan isolation include: a) contamination by other proteins and/or basement membrane components (*i.e.* laminin, fibronectin and type IV collagen) produced by the EHS tumor, b) contamination due to the presence of free GAG chains, and c) degradation of the perlecan core protein.

In the present invention, methods of perlecan purification in quantities appropriate for various assay and therapeutic needs are described. These methods exploit a newly discovered aggregating property of the ~220 kDa PG observed during gel filtration chromatography, which property was not exhibited by perlecan. In addition, the purification protocol of the invention employs a higher pH (preferably pH=8.0), in a higher pH range (pH=7.4 to 8.0+), than conventionally specified (Hassell et al., J. Biol. Chem. 260:8098-8105, 1985; Kato et al., J. Biol. Chem. 262:7180-7188, 1987; Ledbetter et al., Biochem. 26:988-995, 1987), and which higher pH is believed to significantly attenuate or block protein-GAG interactions (such as those described by Heinedgård and Sommarin, Methods Enzym. 144:319-372, 1987), thereby advantageously decreasing the involvement of possible contaminants which might otherwise bind to perlecan during fractionation. The perlecan preparations obtained from this novel methodology are of consistently high quality as demonstrated by a demonstrably intact core protein, and by absence of any significant quantity of contaminating proteins and/or free GAG chains.

Infusion of this perlecan product into rodent brain, in the presence of Aß, consistently leads to fibrillar Aß amyloid deposits in the brain tissue in 100% of tested animals, but which is only observed in 60% of animals following infusion of Aß alone.

Examples

The following examples are set forth to provide those with ordinary skill in the art with the disclosure and description of detailed methodology to obtain "clean" perlecan, and for the isolation of a ~220 kDa heparan sulfate proteoglycan (HSPG) from EHS tumor. However, it should not be construed that the invention is limited to these specific examples.

Materials Used for the Examples Describing the Invention

C57BL mice were purchased from B & K Universal (Kent, WA). Nembutal was from Abbott Laboratories (North Chicago, IL). Alcian blue, Coomassie blue, EHS laminin, bovine plasma fibronectin, EHS type IV collagen, affinity purified rabbit polyclonal anti-laminin (L9393), heparinases I, II and III, BSA, normal goat serum, guanidine-HCl, CHAPS, Tris-HCl, N-ethylmaleimide (NEM), 6-aminohexanoic acid, benzamidine-HCl, phenylmethylsulfonyl fluoride (PMSF), silver nitrate, sodium bicarbonate, sodium nitrite and sodium citrate were all purchased from the Sigma Chemical Company (St. Louis, MO). Methanol, potassium acetate, glutaraldehyde, sodium hydroxide, ammonium hydroxide, sodium azide, formaldehyde, acetic acid, sodium chloride, (ethylenediamine)tetraacetic acid (EDTA), and urea were all from J.T. Baker Inc. (Phillipsburg, N.J.). Absolute ethanol was from McCormick (Pekin, IL). Sephacryl S-400 and S-1000, DEAE-Sephacel and all columns used for analysis were purchased from Pharmacia (Uppsala, Sweden). Triton X-100 was from Boehringer Mannheim (Indianapolis, MN).The conductivity meter with Model number 2052 was from VWR Scientific (Seattle, WA). The mini-protean II electrophoresis system, mini transblot electrophoresis transfer cell, pre-cast polyacrylamide gradient gels (4-15%), electrophoresis running buffer, SDS sample buffer, and pre-stained molecular weight protein standards were from Bio-Rad (Richmond, CA). Nitrocellulose (0.45_m) was from Schleicher and Schuell (Keene, NH). Anti-laminin polyclonal antibody (AB756), and anti-fibronectin polyclonal antibody (AB1941) were Chemicon (Temicula, CA). Biotinylated secondary antibodies (goat anti-rat and goal anti-rabbit) were purchased from Jackson ImmunoResearch (West Grove, PN). Avidin alkaline phosphatase conjugate and alkaline phosphatase substrate solution (Vectastain ABC kit) were from Vector Labs, Inc. (Burlingame, CA). Tween-20 was from Calbiochem Corp. (La Jolla, CA). Anti-perlecan core protein monoclonal antibody (HK-102) was a generous gift from Dr. Koji Kimata (Aichi, Japan).

Example 1

Perlecan Purification from the Engelbreth-Holm-Swarm Tumor

Figure 1 shows a protocol for perlecan purification from the EHS tumor. The EHS tumor was maintained in the right or left hind leg muscle of C57Bl mice following injection of tumor cells as previously described (Swarm, J. Natl. Cancer Inst. 31:953-975, 1963; Swarm et al., J. Natl. Cancer Inst. 33:657-672, 1964; Orkin et al., J. Exp. Med. 145:204-220, 1977). The tumors were usually maintained in the mice hind legs for 3-4 weeks usually attaining a growth of approximately 3-4 grams. In accordance with National Institute of Health (Bethesda, MD, USA) Animal Care and Use Guidelines, the animals were sacrificed by lethal injection of Nembutal (0.50ml of 50mg/ml solution per mouse), before the tumor tissue reached an approximate weight of 4 grams. EHS tumor tissue was harvested from the mice as previously described (Orkin et al., J. Exp. Med. 145:204-220, 1977). All extraction steps (described below) were carried out by agitation using a rotary shaker at 600 rpm (Model M65825, Barnstead/Thermolyne, Dubuque, IA). The tumor tissue (50 grams at a time) was routinely minced and extracted with 2.5 tissue volumes of 50 mM Tris-HCl (pH 7.5), 3.4 M NaCl, containing a protease inhibitor cocktail including 10 mM EDTA, 10 mM NEM, 10 mM 6-aminohexanoic acid, 5.0 mM benzamidine-HCl, and 1mM PMSF.

The supernatants were collected following centrifugation at 17,000Xg for 30 minutes. This high salt extraction was repeated and the resulting pellet obtained was extracted with 2.5 tissue volumes of 4M guanidine-HCl, 0.5% (w/v) CHAPS, 50 mM Tris-HCl (pH 7.5) containing a protease inhibitor cocktail (as described above) for 3 hours at 4°C. The supernatants were again collected following centrifugation at 17,000Xg for 30 minutes. Additional extractions of the remaining pellet were achieved using 1.5 tissue volumes with guanidine-HCl (as described above), first for 2 hours, and then overnight. The guanidine extracts were then pooled and applied (80 mls at a time) to Sephacryl S-400 columns (4.8 X28 cm). Samples were then eluted using a urea buffer containing 7M urea, 0.2M NaCl, 0.1% (w/v) CHAPS, 50 mM Tris-HCl (pH 8.0) containing protease inhibitors as described above. The void volume fractions (believed to contain perlecan) from the Sephacryl S-400 column were then pooled and supplemented with 0.5% Triton X-100 (v/v) and applied to a 50 ml DEAE-Sephacel column packed in a 60 ml plastic syringe equilibrated with urea buffer. Contaminant proteins and non-PGs were removed by first washing the column with 3 column volumes of urea buffer containing 1% Triton X-100, followed by 3 column volumes of urea buffer containing 0.25M NaCl and 1% Triton X-100, and then 3 column volumes of urea buffer containing 0.25 M NaCl without Triton X-100. Bound PGs were then eluted with 5 column volumes of urea buffer containing 1M NaCl or 3M NaCl. The 3M

NaCl elutions were used to determine whether tightly bound PGs not eluted with 1M NaCl still remained bound to the column.

PGs obtained from the DEAE-Sephacel column were first loaded onto a Sephacryl S-500 column (2.6 X 60 cm) in order to try to separate the 220 kDa PG from perlecan (700-800 kDa). In a preliminary study, we found the Sephacryl S-500 column was not able to properly separate the 220 kDa PG from perlecan (both were found in the void volume fractions), due to the self-aggregating ability of the 220 kDa PG. Even the use of dissociating buffers including, a) 4M guanidine-HCl, 0.5% CHAPS, and 50 mM Tris-HCl (pH 7.5), b) 7M urea, 0.2M NaCl, 1% SDS (w/v), and 50 mM Tris-HCl (pH 8.0) and c)7M urea, 0.2M NaCl, 0.1% CHAPS, and 50 mM Tris-HCl (pH 8.0), were not able to prevent the aggregation of the 220 kDa PG. Separation of the 220 kDa PG from perlecan was finally achieved using a Sephacryl S-1000 column (5 X 95 cm) under associating conditions (1M urea buffer containing 50 mM Tris -HCl and protease inhibitors including 1.4 mM EDTA, 1.4 mM NEM, 1.4 mM 6-aminohexanoic acid, 0.7 mM benzamidine, and 0.14 mM PMSF; pH 8.0).

PGs eluted from DEAE-Sephacel were loaded (50 mls per run) onto the Sephacryl S-1000 column (as described above). PGs eluted from the Sephacryl S-1000 column were monitored by SDSPAGE analysis in order to assess the purity of perlecan and other HSPGs produced. For this analysis, 100 µl aliquots of each 60 ml fraction was precipitated with 4 volumes of absolute ethanol by cooling on dry ice for 1 hour, and then centrifuged on a microcentrifuge at 12,000Xg for 20 minutes, and run on SDS-PAGE as described below. Pooled fractions (from 5 separate runs) containing perlecan (K_{av} = 0.29-0.54) were then concentrated on and eluted from a 15 ml DEAE-Sephacel column with 3M NaCl, and rechromatographed onto the Sephacryl S-1000 (as described above). Usually three passes through the Sephacryl S-1000 column gave high quality perlecan preparations free from any other contaminating HSPGs (*i.e.* 220 kDa PG) or other PGs. The perlecan fractions were pooled and concentrated onto a 10 ml DEAE-Sephacel column and ethanol precipitated (as described above). The resulting pellets were dissolved in 3-5 ml of double distilled water and extensively dialyzed against double distilled water until the conductivity of the end product contained very little to no salt (2-3 µmhos) as measured using a digital conductivity meter. The final perlecan product was then freeze-dried and stored. The final purity of the perlecan preparations were further assessed by Alcian blue staining, Coomassie Blue staining, silver staining, and a series of Western blots (as described below).

SDS-PAGE

SDS-PAGE was performed according to the method of Laemmli Laemmli, Nature 227:680-685, 1970) using a Mini-Protean II electrophoresis system with precast 4-15% polyacrylamide gels. In order to examine perlecan quality on SDS-PAGE following the Sephacryl S-1000 chromatography described above, pelleted fractions were redissolved in 20 µl of 1X reducing SDS sample buffer, heated for 5 minutes in a boiling water bath, and electrophoresed at 200V for 45 minutes along with pre-stained molecular weight protein standards. All gel staining and washing steps were carried out with mild agitation using a rotary shaker.

Alcian Blue, Coomassie Blue and Silver Staining

Alcian blue staining of SDS-PAGE gels was employed to detect PGs (Wall and Gyi, Anal. Biochem. 175:298-299, 1988) using a modified method (Castillo and Snow, unpublished observations). Following electrophoresis, gels were rinsed 3 times (for 20 minutes each) with 50% methanol, 10% acetic acid (v/v) and then stained for 2 hours with 0.1% Alcian blue in 50% methanol and 10% acetic acid. For destaining, the gels were rinsed 6-10 times for 20 min each, with the same solution as described above, but without Alcian blue. Non-PG proteins were visualized by staining for 2 hours in the same solution (described above) containing 0.2% (w/v) Coomassie Brilliant Blue and destaining in a similar manner as in Alcian Blue staining protocol (described above).

Some gels were also stained with silver to detect any contaminating proteins in our preparations, using the methods previously described (Oakley et al., Anal. Biochem. 105:361-363, 1980), with slight modifications. Briefly, gels were washed for 25 minutes in 50% methanol and 10% acetic acid (v/v), followed by three washes (10 minutes each) in 10% ethanol and 5% acetic acid (v/v), and then two washes (10 minutes each) with distilled deionized (DD) water. Gels were then fixed for 20-40 minutes with freshly made 1% (v/v) glutaraldehyde and 0.2 M sodium bicarbonate, and washed twice (10 minutes each) with DD water. The gels were then stained for 30 minutes with freshly made 0.8% $AgNO_3$ (w/v), 0.07% NaOH (w/v), 1.3% NH_4OH (v/v) and 15% ethanol (v/v), rinsed three times (10 minutes each) with DD water, and developed using a solution containing 0.005% sodium citrate (w/v), 0.037 % formaldehyde (v/v), and 10% ethanol (v/v). The silver reaction was stopped by adding 5% acetic acid (v/v).

Digestion with Heparitinase/Heparinase and Nitrous Acid

Prior to SDS-PAGE, some of the ethanol precipitated HSPGs were digested by incubation overnight at 41°C with 1.0 Unit each of heparinases I, II and III

(heparitinase) in 90µl of digestion buffer consisting of 100 mM Tris-HCl, 2.5 mM calcium acetate, 5.0 mM 6-aminohexanoic acid, 2.5 mM benzamidine-HCl, 5.0 mM NEM, and 0.50 mM PMSF (pH 7.0). The next day, digested samples were precipitated by adding 3.5 volumes of 95% ethanol, 1.5% potassium acetate (w/v), cooled on dry ice for 1 hour and then centrifuged at 12,000 X g for 20 minutes. For Western blotting of perlecan core protein, the pellets were redissolved in 20µl of non-reducing SDS sample buffer (since reduction eliminates the antigenic sites recognized by anti-HK-102)(Kato et al., J Cell Biol. 106:2203-2210, 1988).

In addition, prior to SDS-PAGE, some of the ethanol precipitated HSPG samples were digested with nitrous acid (1 µl/ 1 µg HSPG) using $NaNO_2$ in 1.8M acetic acid at a final concentration of 0.24 M (44). Following a 20 minute nitrous acid digestion at room temperature, 5 volumes of absolute ethanol was added to the reaction mixtures, vortexed and centrifuged at 14,000 X g for 20 minutes. The pellets were then dissolved in 1X SDS-PAGE sample buffer for electrophoretic separation and detection of the liberated perlecan core protein. Silver staining of heparitinase/heparinase and nitrous acid digested samples was then employed as described above.

Analysis of Final Perlecan Preparations by Western Blotting

SDS-PAGE was performed as described above and the separated proteins were transferred to nitrocellulose using a Mini transblot electrophoresis transfer cell. Electrotransfer was performed at 100V for 2 hours. Following transfer, membranes were rinsed with water and blocked overnight with 0.15% (w/v) bovine serum albumin, 1% (v/v) normal goat serum, 100 mM Tris-HCl, and 3mM NaN_3 (pH 7.4). Nitrocellulose membranes probed with fibronectin antibody were blocked in the same solution described above without normal goat serum. Blots were probed with a monoclonal antibody (used at a 1:750 dilution) against perlecan core protein (HK-102), b) an affinity-purified polyclonal antibody (used at a 1:2000 dilution) against laminin, c) a polyclonal antibody (used at a 1:25,000 dilution) against fibronectin (AB1941) and d) a polyclonal antibody (used at a 1:25,000 dilution) against type IV collagen. The primary antibodies (described above) were diluted with Tris-buffered saline containing 100 mM Tris-HCl, 50 mM NaCl, 0.05% Tween-20, and 3 mM NaN_3 (pH 7.4)(TTBS). Corresponding blots were incubated with primary antibodies for three hours, washed with TTBS three times (10 minutes each), followed by a 1 hour incubation with the appropriate biotinylated secondary antibodies diluted 1:1000 with TTBS. The membranes were then rinsed three times (10 minutes each) with TTBS, probed for 30 minutes with avidin alkaline phosphatase conjugate (Vectastain), rinsed again (as

described above), followed by the addition of an alkaline phosphatase substrate solution (Vectastain). Following color development, the reaction was stopped by flushing the membranes with DD water.

Example 2

Analysis at Each Step of the Perlecan Isolation Procedure

In order to determine the effectiveness of our perlecan isolation protocol, the presence of perlecan, other PGs and non-PG proteins were monitored at each step of the isolation method (Figure 1). As shown in Figure 2 lane 1, the 4M guanidine-HCl extracts contained two major PGs which were detected by Alcian blue staining (blue bands). These included a high M_r PG, believed to represent perlecan (at the interface of the resolving gel), and a 220 kDa PG, believed to represent the high density HSPG, previously reported (Fujiwara et al., Eur. J. Biochem. 143:145-157, 1984; Hassell et al., J. Biol. Chem. 260:8098-8105, 1985). The guanidine-HCl extraction protocol recovered > 90% of GAGs as determined using an quantitative Alcian blue staining assay (Björson, Anal. Biochem. 210:282-291, 1993).

In addition to the two major PGs (described above), the guanidine-HCl extracts contained many non-PG proteins as shown by Coomassie blue staining (purple stained bands in Figure 2, lane 1). The guanidine extracts were then pooled and applied to Sephacryl S-400 columns. Figure 2, lane 2, demonstrates the presence of the two major PGs (detected by Alcian blue) and non-PG proteins (detected by Coomassie blue) following elution from Sephacryl S-400 columns. Gel filtration chromatography using Sephacryl S-400 columns allowed for both buffer exchange and removal of many (but not all) of the non-PG proteins (compare the Coomassie blue stained bands in Figure 2, lane 2 to Figure 2, lane 1). The void volume fractions from the Sephacryl S-400 columns were then pooled and supplemented with 0.5% Triton X-100 (v/v) and applied to a DEAE-Sephacel column equilibrated with urea buffer.

Subsequent washing of the DEAE column with urea buffer containing Triton X-100 and 0.25M NaCl removed all of the unbound non-PG proteins, as no Coomassie-blue stained bands were apparent in the last portion of the 0.25M NaCl eluate (Figure 2, lane 3). The subsequent 1.0 M NaCl elution from the DEAE-Sephacel column removed the two major PGs present (*i.e.* believed to represent perlecan and the 220 kDa PG) and demonstrated the absence of any non-PG proteins (by lack of positive Coomassie blue stained bands) (Figure 2, lane 4). Further elution with 3M NaCl demonstrated the absence of any residual PGs or non-PG proteins bound to the DEAE

column (Figure 2, lane 5) and indicated that the majority of PGs were removed from the DEAE column using 1.0 M NaCl.

In order to completely separate the 220 kDa PG component from perlecan (shown in Figure 2, lane 4) gel filtration chromatography using a Sephacryl S-500 was evaluated. It was expected that fractionation using a Sephacryl S-500 would separate perlecan (expected to elute in the void volume) from the 220 kDa PG (expected to elute with a K_{av} of ~ 0.45). However, under these conditions the 220 kDa PG apparently aggregated and eluted in the void volume, with perlecan eluted shortly thereafter (with a $K_{av} = 0.20$). In order to achieve better separation between perlecan and the 220 kDa PG, a variety of different dissociating eluants were tried. These eluants included 4 M guanidine-HCl with 0.1% CHAPS, 7M urea with 0.1% CHAPS, and 7M urea with 1% SDS. Regardless of the eluants employed (as described above), the 220 kDa PG was always present in the void volume fractions and was present in most fractions containing perlecan. Separation of perlecan from the aggregating 220 kDa PG was achieved using a Sephacryl S-1000 column. As shown (Figure 2, lanes 6 and 7; and Figure 3), this column was effective in separating perlecan from the 220 kDa PG, which under these conditions still aggregated and was primarily found in the void volume fractions with a K_{av} <0.33 (Figure 3). Perlecan was now effectively separated from the 220 kDa PG and was primarily present in fractions with a $K_{av} = 0.20\text{-}0.70$, with a major peak at $K_{av} = 0.40$ (Figure 3). Perlecan could therefore be successfully isolated without contamination by the 220 kDa PG by pooling fractions with a $K_{av} = 0.37\text{-}0.54$ (Fig. 2, lane 7 and Figure 3). The 220 kDa PG (with only slight perlecan contamination) could also be isolated by pooling fractions with a $K_{av} = 0.0 - 0.37$ (Figure 2, lane 6 and Figure 3). Any lower molecular weight GAGs observed in the pooled perlecan fractions following a second pass through the Sephacryl S-1000 column (Fig. 2, lane 7; Fig. 3A) were subsequently removed following a third pass through the Sephacryl S-1000 column, yielding a pure perlecan product (see Figs. 4 and 5).

Example 3

Assessment of Purity of Final Perlecan Preparations by Silver and Alcian Blue Staining

The purity of perlecan that was obtained in the final preparations were evaluated for quality. For these analyses, a combination of silver staining, alcian blue staining and western blotting were employed (as described below) on aliquots of purified perlecan. In order to determine that no significant quantity of other proteins were present in our final perlecan preparations, silver staining of SDS-PAGE gels were

first used. For these studies, 6.25 μg aliquots (determined by Lowry) of perlecan were separated on 4-12% gradient SDS-PAGE gels under reducing conditions and stained with silver (see Materials and Methods). As shown in Figure 4A, perlecan was observed at the resolving gel interface, with no other lower M_r bands present. This observation indicated that our final perlecan preparations did not contain any contaminating proteins with a M_r < 700-800 kDa (detectable by silver staining).

This same perlecan preparation was then stained with Alcian blue (Figure 4B) to determine possible contamination by other smaller PGs and/or free GAG chains. As shown in Figure 4B, lane 1, Alcian blue staining of undigested perlecan demonstrated only one band (indicative of intact perlecan) at the 4-12% gradient SDS-PAGE gel interface. No other Alcian blue stained bands of lower M_r were observed indicating the absence of any other PGs and/or free GAG chains in our final perlecan preparations. To confirm that the high M_r Alcian blue stained material (believed to represent perlecan)(Figure 4B, lane 1) contained heparan sulfate GAGs, digestion with heparinase/ heparitinase (specific for heparan sulfate and/or heparin GAGs) was also used. As shown in Figure 4B, lane 2, following digestion with heparitinase / heparinase a marked reduction in Alcian blue staining was observed (compare Fig. 4B, lane 2 to lane 1). This indicated that the putative perlecan band (shown in Fig. 4B, lane 1) contained heparan sulfate GAG chains.

In order to demonstrate that the Alcian blue and silver stained bands observed at the resolving gel interface in Fig. 4A (lane 1) and 4B (lane 1) were in fact intact perlecan, and that no other proteins or contaminating PGs were present in the final perlecan preparations, heparitinase/heparinase and nitrous acid digested samples of the final perlecan product were also stained with silver (Fig. 5). As shown in Fig. 5, lane 1 (arrow) a discrete doublet characteristic of the perlecan core protein was observed at ~400 kDa by silver staining following heparitinase/ heparinase digestion. Silver staining of only the heparitinase/ heparinase enzymes employed (in the absence of perlecan)(Fig. 5, lane 2) did not show the characteristic ~400 kDa doublet observed in Fig. 5, lane 1. Nitrous acid digestion of the final perlecan product followed by silver staining also yielded similar results (Fig. 5, lane 3). A characteristic ~400 kDa band indicative of the intact perlecan core protein was observed on silver staining following nitrous acid pretreatment (Fig. 5, lane 3).These studies indicated that no other protein or PG bands were present in the final perlecan preparations, even following liberation of the perlecan core protein by heparitinase/heparinase or nitrous acid.

Example 4

Assessment of Purity of Final Perlecan Preparations by Western Blotting

In order to further demonstrate and confirm the presence of perlecan, and the absence of other basement membrane components produced by the EHS tumor (*i.e.* laminin, fibronectin and type IV collagen) in the final perlecan preparations, Western blotting with specific antibodies was employed. Figure 6, lane 1 is a Western blot of our final perlecan preparation following separation on a 4-12% gradient SDS-PAGE (under non-reducing conditions) probed with a monoclonal antibody against perlecan core protein. As shown, perlecan immunoreactivity was apparent at the resolving gel interface, proving that the positive silver (Fig. 4A) and Alcian blue (Fig. 4B, lane 1) staining observed at the resolving gel interface previously, was in fact intact perlecan core protein. Following heparinase/ heparitinase digestion (Fig. 6, lane 2) the perlecan monoclonal antibody detected a doublet at 400 kDa and 360 kDa, characteristic and indicative of intact perlecan core protein (Hassell et al, J. Biol. Chem. 260:8098-8105, 1985; Ledbetter et al., Biochem. 26:988-995, 1987; Kato et al, J. Cell Biol. 106:2203-2210, 1988).

The purity of the final perlecan preparations were further confirmed by Western blotting and probing with a series of antibodies directed against other basement membrane components known to be produced by the EHS tumor and known to bind to perlecan. As shown in Figure 6, lane 3, although 1μg of EHS laminin was strongly stained on Western blot when probed with an anti-laminin antibody (positive control for Figure 6, lane 4), no positive immunostaining for laminin was observed in 6.25 μg of our final perlecan preparation (Figure 6, lane 4). Similarly, although 1μg of fibronectin stained strongly on Western blot when probed with an anti-fibronectin antibody (Figure 6, lane 5), no positive immunostaining for fibronectin was observed in 6.25 μg of our final perlecan preparation. Furthermore, although 1 μg of type IV collagen stained strongly on Western blot when probed with an anti-type IV collagen antibody (Figure 6, lane 7), no positive Immunostaining for type IV collagen was observed in 6.25 μg of our final perlecan preparation (Figure 6, lane 8). These latter studies indicated that only perlecan, and no other basement membrane components produced by the EHS tumor, was present in the final perlecan preparations.

Example 5

Discussion Pertaining to Perlecan Isolation Methodology

The present invention discloses methods for isolation of perlecan from the EHS tumor. The protocol is unique in part in that the method does not require cesium chloride density gradient centrifugation, as has been previously specified by all

investigators (Fujiwara et al., Eur. J. Biochem. 143:145-157, 1984; Hassell et al, J. Biol. Chem. 260:8098-8105, 1985; Ledbetter et al., Biochem. 26:988-995, 1987; Paulsson et al., J. Mol. Biol. 197:297-313, 1987; Kato et al, J. Cell Biol. 106:2203-2210, 1988). The perlecan product obtained by our protocol was of high quality and did not contain any contaminants produced by the EHS tumor, including other basement membrane components (*i.e.* laminin, fibronectin, and type IV collagen)(assessed by silver staining and Western blotting with specific antibodies) and/or free GAG chains (assessed by Alcian blue staining). The presence of contaminating basement membrane proteins (especially laminin) in perlecan preparations has posed a major problem in the past, and it is essential that the perlecan used for *in vitro* and/or *in vivo* studies be thoroughly analyzed for components which can potentially interact with perlecan during the isolation procedure. In the present invention, a detailed perlecan isolation procedure employed for consistent production of high quality perlecan is described. In addition, the quality control steps employed to ensure the production of an essentially clean perlecan product are disclosed.

Comparison to Known Perlecan Isolation Methods

Isolation of perlecan has been previously described by several investigators (Fujiwara et al., Eur. J. Biochem. 143:145-157, 1984; Hassell et al, J. Biol. Chem. 260:8098-8105, 1985; Ledbetter et al., Biochem. 26:988-995, 1987; Paulsson et al., J. Mol. Biol. 197:297-313, 1987; Kato et al, J. Cell Biol. 106:2203-2210, 1988). In one previous study (Hassell et al, J. Biol. Chem. 260:8098-8105, 1985), perlecan was isolated from EHS tumor initially using nondenaturing conditions (extraction with buffered saline), followed by denaturing conditions (extraction with urea). The urea extracts were then processed utilizing DEAE-Sephacel chromatography, followed by ultracentrifugation, dialysis, gel filtration chromatography (Sepharose CL-4B), exhaustive dialysis and lyophilization. One of the problems noted by the authors in the above procedure (Hassell et al, J. Biol. Chem. 260:8098-8105, 1985) was the contaminating presence of laminin and other proteins, believed to be inherently bound to perlecan by disulfide-dependent association, which could only be removed by disulfide bond reduction (using dithiothreitol) followed by ion exchange chromatography. In another study, increased purity was achieved when the protocol was modified by pre-extracting the tumor tissue with 3.4 M NaCl before extraction with 6M urea (Ledbetter et al., Biochem. 26:988-995, 1987). However, this latter method resulted in occasional partial degradation of the large 400 kDa perlecan core protein (Paulsson et al., J. Mol. Biol. 197:297-313, 1987).

Extraction of EHS tumor with chaotrophic agents such as 4M guanidine-HCl allowed for rapid and efficient extraction of PGs (Hassell et al., Proc. Natl. Acad. Sci. 77:4494-4498, 1980; Kato et al., J. Biol. Chem. 262:7180-7188, 1987; Kato et al, J. Cell Biol. 106:2203-2210, 1988) and is the preferred extraction protocol for the invention, although others may be made to serve as well. The denaturing conditions and the use of protease inhibitors in the extraction solution protect the integrity of the large perlecan core protein (see Fig. 2, lane 1), which is susceptible to proteolysis (Paulsson et al., J. Mol. Biol. 197:297-313, 1987). It is calculated that >90% of GAGs (as determined by an Alcian blue assay) (Björnson, Anal. Biochem. 210:282-291, 1993) were extracted from EHS tumor tissue using 4M guanidine-HCl containing 0.5% CHAPS and protease inhibitors (not shown).

However, in addition to the presence of PGs, the guanidine extracts contained large amounts of non-PG proteins which may potentially interfere with subsequent ion-exchange chromatography. Gel filtration chromatography using a Sephacryl S-400 column allowed for both buffer exchange (7M urea with detergent and protease inhibitors) and the removal of a large number of small molecular weight proteins (compare Fig. 2, lane 2 to lane 1) which could not be accomplished by a much longer dialysis process, contrary to what has been previously suggested (Kato et al., J. Biol. Chem. 262:7180-7188, 1987; Hassell et al, J. Biol. Chem. 260:8098-8105, 1985). The guanidine-HCl extract from 50 grams of EHS tumor (~ 400 ml) was exchanged into 7M urea buffer (described above) in less than one day, requiring less than 3 liters of buffer.

Yield of Perlecan

The final yield of perlecan using the method of the invention was approximately 10-12.5 μg per gram of EHS tumor wet weight, as measured by protein determination (Hassell et al., Proc. Natl. Acad. Sci. 77:4494-4498, 1980). Although clonal differences in EHS tumor may result in variations in GAG chain length (Paulsson et al., J. Mol. Biol. 197:297-313, 1987), a protein to GAG ratio of approximately 1:1 in our purified perlecan product was calculated (not shown). This is similar to GAG:protein ratios of the low density HSPG (*i.e.* perlecan) previously described by Fujiwara et al (Eur. J. Biochem. 143:145-157, 1984). Additionally, the total GAG content present in the EHS tumor tissue determined by using an Alcian blue assay (Björnson, Anal. Biochem. 210:282-291, 1993), was approximately 0.90 mg of GAGs per gram wet weight of EHS tumor (not shown). This is greater than the 0.75 mg of GAGs per gram wet weight of EHS tumor previously reported by Fujiwara et al (Eur. J. Biochem. 143:145-157, 1984). Following S-1000 fractionation, only 20% (150-180μg per gram tumor wet weight) of

the calculated GAG content was found to be attributed by perlecan, consistent with the study by Dziadek et al (EMBO J 4:905-912, 1985), whereas most of the remaining GAG content was present in the 220 kDa PG (not shown). Based on these determinations, the final perlecan yield using the described protocol from starting EHS tumor tissue was approximately 10%. Two hundred grams of EHS tumor consistently yields approximately 3-4 mg of perlecan.

Example 6

Identification of an Aggregating ~220 kDa PG Which Can Be Separated From Perlecan

Earlier studies have demonstrated two major PGs to be produced by the EHS tumor (Fujiwara et al., Eur. J. Biochem. 143:145-157, 1984; Dziadek et al., EMBO J 4:905-912, 1985 ; Kato et al., J. Biol. Chem. 262:7180-7188, 1987; Hassell et al, J. Biol. Chem. 260:8098-8105, 1985; Ledbetter et al., Biochem. 26:988-995, 1987). Based on cesium chloride density gradient centrifugation they were designated as a high density HSPG (≥1.65 g/ml) and a low density HSPG (1.38 g/ml)(Kato et al., J. Biol. Chem. 262:7180-7188, 1987; Hassell et al, J. Biol. Chem. 260:8098-8105, 1985; Ledbetter et al., Biochem. 26:988-995, 1987). The low density HSPG was later found to be analogous to perlecan, whereas the high density HSPG had an electrophoretic mobility of 200-400 kDa (Fujiwara et al., Eur. J. Biochem. 143:145-157, 1984), and was postulated to represent either a breakdown product of the low density HSPG (*i.e.* perlecan)(Hassell et al, J. Biol. Chem. 260:8098-8105, 1985; Ledbetter et al., Biochem. 26:988-995, 1987), or an independent PG derived from a separate gene product (Kato et al., J. Biol. Chem. 262:7180-7188, 1987; Paulsson et al., J. Mol. Biol. 197:297-313, 1987). Although these latter possibilities have not been fully resolved, the high density HSPG previously identified by cesium chloride centrifugation is most likely identical to the 220 kDa PG described herein. This 220 kDa PG, like the known high density HSPG (Kato et al., J. Biol. Chem. 262:7180-7188, 1987; Hassell et al, J. Biol. Chem. 260:8098-8105, 1985; Ledbetter et al., Biochem. 26:988-995, 1987), was found to be the major PG extractable by saline and contained heparan sulfate GAG chains as demonstrated by degradation with heparinase/ heparitinase digestion (not shown).

One of the unique discoveries of the present invention is that under certain chromatographic conditions, the 220 kDa PG self-aggregates, which enables it to be separated from perlecan (which itself did not aggregate under similar conditions). These differences in aggregating properties allowed us to develop a novel method to purify perlecan without contamination by the 220 kDa PG, using Sephacryl S-1000

chromatography. The self-aggregating property of the 220 kDa PG appeared not to involve any other proteins as determined by analysis of SDS-PAGE gels by silver staining (not shown). The 220 kDa PG eluted from the Sephacryl S-1000 in the void volume (see Fig. 3) indicating that this PG formed large aggregates of >>100,000 kDa, which occurred even under dissociating conditions.

Example 7

Further Improved Protocol for Perlecan Isolation from EHS Tumor

Figure 7 describes an alternate protocol used for perlecan isolation from the EHS tumor, which contains two additional steps which both further improve perlecan yield and reduce the number of passes required for Sephacryl S-1000 chromatography.

The first improvement to the method disclosed in detail in Examples 1 - 4, is the use of a Sulphopropyl Sepharose (SP) column, a cationic exchange column which is also effective in removing any contaminating proteins which may interact with perlecan during the isolation procedure. For this step, the void volume fractions (believed to contain perlecan) from the Sephacryl S-400 column are pooled and supplemented with 0.5% Triton X-100 (v/v) and applied to a 50 ml Sulphopropyl Sepharose (SP) column (Pharmacia) packed in a 60 ml plastic syringe. The eluates are then applied to the 50 ml DEAE-Sephacel column and follow the corresponding procedure as detailed in Examples 1 - 4. It is an aspect of the invention that cationic exchange columns (*i.e.* anionic resins) such as DEAE-Sephacel can be employed to clean up PG extracts by removing unwanted proteins or macromolecules which associate with the PGs *in vivo* and/or during the isolation procedure.

A second improvement to the method disclosed in Examples 1 - 4 is the use of a heparin-Sepharose column which is also effective in removing possible contaminating laminin, fibronectin, and/or type IV collagen, all of which interact with perlecan during the isolation procedure. For this step, PGs eluted from DEAE-Sephacel are precipitated with 4 volumes of methanol containing 2.5% (w/v) sodium acetate by cooling on dry ice for 1 hour and then centrifuging at 17,000Xg for 20 minutes. The pellet is redissolved in 50 ml of 1M urea buffer (containing 50 mM Tris-HCl and protease inhibitors including 1.4 mM EDTA, 1.4 mM NEM, 1.4 mM 6-aminohexanoic acid, 0.7mM benzamidine, and 0.14 mM PMSF; pH 8.0) and loaded onto the Sephacryl S-1000 column (as described in Examples 1 - 4). The eluate containing perlecan (K_{av} = 0.29-0.54) is redirected through an 8 ml heparin-Sepharose column (Pharmacia) before going to the fraction collector. The fractions were then monitored by SDS-PAGE

analysis in order to access the purity of the perlecan and other heparan sulfate PGs which may be present.

For this latter analysis, 100 µl aliquots of each of 60 ml fractions were precipitated with 4 volumes of absolute ethanol containing 2.5% (w/v) sodium acetate by cooling on dry ice for 1 hour, centrifuged on a microcentrifuge at 12,000Xg for 20 minutes, and run on SDS-PAGE. Fractions containing high quality perlecan free from any other contaminating HSPGs (*i.e.* 220 kDa PG) or other PGs were pooled and precipitated as above. Fractions containing perlecan with other contaminants were also precipitated and passed through a smaller Sephacryl S-1000 column (2.6 X 60 cm) for a second time (as described above). The resulting pellets containing "clean" perlecan were dissolved in 3-5 ml of double distilled water and extensively dialyzed against double distilled water until the conductivity of the end product contained very little to no salt (2-3 µmhos) as measured using a digital conductivity meter. The final perlecan product was then freeze-dried and stored. The final purity of the perlecan preparations were further assessed by Alcian blue staining, Coomassie blue staining, silver staining, and a series of Western blots (as described in sections 4.2.2-4.2.5).

Columns containing immobilized GAGs (such as heparin Sepharose) in an affinity type column chromatography can also be advantageously used to "clean" up PG preparations. For example, a Sepharose column containing immobilized chondroitin sulfate may be used for the isolation of chondroitin sulfate containing PGs from tissues and/or cell culture. Using this methodology, chondroitin sulfate binding proteins or macromolecules will bind to the chondroitin sulfate column when passed through, allowing for "cleaner" preparations of the resulting chondroitin sulfate PGs/GAGs which follow through the column. These columns containing immobilized GAGs may also be advantageously used in large scale for isolation of different types and/or classes of PGs/GAGs for commercial utilities.

Using these two modifications as described above, 200 grams of EHS tumor wet weight has a perlecan yield in the range of 4-5 mg (as compared to 3-4 mg using the protocol described in Examples 1 - 4). In addition, only 2 passes through the Sephacryl S-1000 column (instead of three) are required to obtain "clean" perlecan.

Example 8

Use of the Perlecan Product in a Rat Infusion Model

The clean perlecan and HSPG products obtained from the methodology described in the present invention can then be advantageously used to produce a significantly more consistent and more reliable animal model with which to study

fibrillar amyloid, and in particular Aß amyloid, deposition/persistence in brain than has previously been possible with less pure product. These improved animal models can also be used to screen and identify new therapeutic agents which target fibrillar Aß deposition, accumulation, and/or persistence. As an example to demonstrate the utility of clean perlecan the following study was implemented.

For rat animal model studies, Aß (residues 1-40: lot #WM365; Bachem California Inc., Torrance, CA) was initially dissolved in double distilled sterile water at a concentration of 1 mg/ml (stock solution). 50 µl of Aß stock solutions were then transferred with sterile pipettes to microcentrifuge tubes containing either 50 µl of sterile distilled water, or 25 µg of perlecan recently dissolved in 50 µl of distilled sterile water. The Aß (1-40), and Aß (1-40) + perlecan solutions were then either frozen at -70°C or used immediately in the animal model (described below).

20 male Harlan Sprague-Dawley rats (250-300 g; 3 months old) were anesthetized with pentobarbital (50 mg/kg) and a 27 gauge stainless steel cannula was stereotactically implanted into the hippocampus using bregma as reference point (AP -4.8; ML 3.5; DV 3.0) and secured to the skull by machine screws and dental acrylic. The cannula was connected via a 15 cm coil of vinyl tubing to a model 2002 osmotic minipump (Alzet Inc.) placed subcutaneously beneath the shoulder blades. The infused solution was contained entirely within the coil of vinyl tubing and separated from water in the pump (dyed blue with food coloring) by a 3 cm air spacer. Successful performance of the pumps was confirmed by measuring movement of the air spacer and blue saline solution following sacrifice. In the present study to demonstrate the effects of isolated EHS perlecan in this animal model, 10 animals received infusion of either Aß (1-40) only, or Aß (1-40) + Perlecan, directly into hippocampus at a flow rate of 0.5 µl/hr for 1 week. A high concentration of Aß was chosen to maximize possible effects following infusion into brain. The quantity of Aß peptide infused into brain by the end of 1 week in each animal was approximately 50 µg.

Rats were sacrificed by an overdose of pentobarbital and perfused with 100 ml of saline followed by 150 ml of 4% paraformaldehyde buffered with phosphate (pH 7.4), the brains were removed and postfixed for 48 hr, and transferred to PBS for frozen tissue sectioning. Consecutive 25 µm serial sections were cut using a sliding microtome and placed on gelatin-coated slides.

As we previously described (Snow et al., Neuron 12:219-234, 1994), detection of infused Aß was monitored using a polyclonal antibody against synthetic Aß or a monoclonal antibody (6E10; Senetek) which recognizes residues 1-17 of Aß. Perlecan

accumulation was monitored using a polyclonal antibody against the core protein of perlecan. From each animal, 100 consecutive serial sections were cut and stained with cresyl violet to identify the area occupied by the infusion site. Usually, the infusion site spanned 40-60 serial sections. Congo red staining (Puchtler et al., J. Histochem. Cytochem. 10:355-364, 1962) and Thioflavin S fluorescence (Elghetany and Saleem, Stain Tech. 63:201-212, 1988) were then used on every 10th section spanning through the entire infusion site to determine the extent and consistency of possible fibrillar Aß amyloid deposition in these animals. The percent of animals containing congophilic deposits (indicative of fibrillar amyloid) in each of the two groups was assessed by blind scoring of tissue sections (scoring of every 10th congo red stained sections through the entire infusion site) using an arbitrary scale of scoring (from 0 to 5) as previously described (Snow et al., Neuron 12:219-234, 1994). Tissue sections with the anti-Aß antibodies were pretreated for 3-5 minutes with 88% formic acid before immunostaining to aid in unmasking hidden antigenic sites as previously reported (Kitamoto et al., Lab. Invest. 57:230-236, 1987). For immunostaining, negative controls consisted of using TBS instead of the primary antibody and/or preabsorption experiments using the primary antibody in the presence of excess antigen (Snow et al., Am. J. Path. 137:1253-1270, 1990).

Results of Aß Only versus Aß + Perlecan Infusion into Rodent Brain

High quality "clean" perlecan is believed to be essential to establish a consistent and reproducible animal model to study the effects of fibrillar Aß amyloid deposits in rodent brain. Following a 1 week-infusion of Aß (1-40) alone, or Aß (1-40) + clean perlecan into rodent hippocampus, it was evident that differences between the two groups existed in the extent and percent of animals with Aß fibrillar amyloid deposits. 100% (10 of 10) of animals infused with Aß (1-40) + clean perlecan for 1 week demonstrated Congo red and Thioflavin S-positive deposits (indicative of amyloid) at the infusion site (Fig. 8). In comparison only 60% (6 of 10) of animals infused with Aß (1-40) alone demonstrated Congo red or Thioflavin S-positive deposits at the infusion site. The Congo red (not shown) and Thioflavin S (Fig. 8B) positive deposits in the Aß + perlecan group corresponded on adjacent serial sections to precisely those areas containing both Aß (Fig. 8B) and perlecan (not shown), as previously demonstrated (Snow et al., Neuron 12:219-234, 1994). This in vivo study therefore demonstrated that consistent fibrillar Aß deposition and persistence could be obtained with the use of a high quality perlecan product (in the presence of Aß 1-40).

Our studies utilizing this animal model suggest that the final purity of the perlecan preparation is essential, if not critical, for continued reliability of fibrillar Aß amyloid deposition and persistence in brain. Contaminating proteins such as laminin, fibronectin and type IV collagen, which are produced by the EHS tumor and which normally associate with perlecan can cause unexpected variability in the animal model (Snow, Cummings and Castillo, unpublished observations). Other investigators who attempt to replicate these findings with the described rodent model must ensure that the perlecan used is of the highest quality and "free" of contaminating proteins, as described in the present invention. Even the presence of free GAG chains (*i.e.* heparan sulfate) in perlecan preparations will compete with perlecan for binding sites on Aß and will disrupt the consistency of this animal model (Snow and Castillo, unpublished observations).

Further Aspects and Utilizations of the Invention

One aspect of the present invention is to provide methods for the consistent production of "clean" and "non-contaminated" perlecan for use in a number of different and relevant *in vitro* and *in vivo biological* assays as described herein.

Improved Methodologies to Isolate Substantially "Pure" Proteoglycans

In our methodology a cationic exchange column (*i.e.* anionic resin) was used and found effective in removing any contaminating proteins which may interact with perlecan during the isolation procedure. For our protocol, we used a 50 ml Sulphopropyl Sepharose (SP) column (Pharmacia) packed in a 60 ml plastic syringe. Cationic exchange columns may be employed to clean up any PG extracts by removing unwanted proteins or macromolecules which associate with the PGs *in vivo* and/or during the isolation procedure. Such strong cationic-exchange resins can be purchased from Pharmacia Biotech (USA) and include columns containing: 1) Mini S (minibeads), 2) Mono S (monobeads), 3) Source 15S (prepacked columns), 4) Source 30S (lab packs), 5) SP Sepharose High Performance, and 6) SP Sepharose Fast Flow. Other anionic resins obtained from other companies may also be useful.

Another improvement to our isolation methodology was the use of a heparin-Sepharose column which was effective in removing possible contaminating laminin, fibronectin, and/or type IV collagen, all of which interact with perlecan during the isolation procedure. For this step in the isolation protocol, the eluate containing perlecan (K_{av} = 0.29-0.54) was re-directed through an 8 ml heparin-Sepharose column (Pharmacia) before going to the fraction collector. Columns containing immobilized GAGs (such as heparin Sepharose) can be also used to "clean" up other types of PG

preparations. For example, a Sepharose column containing immobilized chondroitin sulfate may be useful for the isolation of chondroitin sulfate containing PGs from tissues and/or cell culture. Using this methodology, chondroitin sulfate binding proteins or macromolecules, will bind to the chondroitin sulfate column when passed through, allowing for "cleaner" preparations of the chondroitin sulfate PGs/GAGs which will flow through the column. Similarly, Sepharose columns containing immobilized dermatan sulfate, keratan sulfate, or heparan sulfate may also be used to aid in the isolation of dermatan sulfate PGs, keratan sulfate PGs and heparan sulfate PGs, respectively. These and other immobilized-GAG columns may also be used in large scale for isolation of different types and/or classes of PGs/GAGs for commercial utilities.

Antibodies

One aspect of the present invention is to use perlecan or the ~220 kDa HSPG obtained by the described isolation protocols described herein, to produce new polyclonal and/or monoclonal antibodies. Antibodies generated against "clean" perlecan or the ~220 kDa HSPG, isolated as described herein, can be obtained by direct injection into an animal or by administering to an animal, preferably a nonhuman.

The term "antibody" is meant to include polyclonal antibodies, monoclonal antibodies, chimeric antibodies, anti-idiotypic antibodies to antibodies specific for perlecan or the ~220 kDa HSPG of the present invention.

Polyclonal antibodies are heterogeneous populations of antibody molecules derived from the sera of animals immunized with an antigen.

A monoclonal antibody contains a substantially homogeneous population of antibodies specific to antigens, which population contains substantially similar epitope binding sites. For preparation of monoclonal antibodies, any technique which provides antibodies produced by continuous cell line cultures can be used. Examples include the hybridoma technique (Kohler and Milstein, Nature 256:495-497, 1975), the trioma technique, the human B-cell hybridoma technique (Kozbor et al, Immunology Today 4:72, 1983), and the EBV-hybridoma technique to produce human monoclonal antibodies (Cole et al, in Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp.77-96, 1985). Such antibodies may be of any immunoglobulin class including IgG, IgM, IgE, IgA, GILD and any subclass thereof.

Chimeric antibodies are molecules different portions of which are derived from different animal species, such as those having variable region derived from a murine monoclonal antibody and a human immunoglobulin constant region, which are primarily used to reduce immunogenicity in application and to increase yields in

production. Chimeric antibodies and methods for their production are known in the art (ex. Cabilly et al, Proc. Natl. Acad. Sci. U.S.A. 81:3273-3277, 1984; Harlow and Lane: Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory 1988).

An anti-idiotypic antibody is an antibody which recognizes unique determinants generally associated with the antigen-binding site of an antibody. An anti-idiotypic antibody can be prepared by immunizing an animal of the same species and genetic type (e.g., mouse strain) as the source of the monoclonal antibody with the monoclonal antibody to which an anti-idiotypic antibody is being prepared. The immunized animal will recognize and respond to the idiotypic determinants of the immunizing antibody by producing an antibody to these idiotypic determinants (the anti-idiotypic antibody). See, for example, U.S. Patent No. 4,699,880, which is herein incorporated by reference.

The term "antibody" is also meant to include both intact molecules as well as fragments thereof, such as, for example, Fab and $F(ab')_2$, which are capable of binding antigen. Fab and $F(ab')_2$ fragments lack the Fc fragment of intact antibody, clear more rapidly from the circulation, and may have less non-specific tissue binding than an intact antibody (Wahl et al, J. Nucl. Med. 24:316-325, 1983).

The antibodies or fragments of antibodies, useful in the present invention may be used to quantitatively or qualitatively detect perlecan, perlecan-derived fragments or the ~220 kDa HSPG (and its fragments) in a sample or to detect presence of cells which express perlecan or the ~220 kDa HSPG. This can be accomplished by immunofluorescence techniques employing a fluorescently labeled antibody coupled with light microscopic, flow cytometric or fluorometric detection.

One of the ways in which a perlecan, perlecan-fragment or ~220 kDa HSPG antibody can be detectably labeled is by linking the same to an enzyme and use in an enzyme immunoassay (EIA). This enzyme, in turn, when later exposed to an appropriate substrate, will react with the substrate in such a manner as to produce a chemical moiety which can be detected, for example, by spectrophotometric, fluorometric, or by visual means. Enzymes which can be used detectably label the antibody include, but are not limited to, malate dehydrogenase, staphylococcal nuclease, delta-5-steroid isomerase, yeast alcohol dehydrogenase, alpha-glycerophosphate dehydrogenase, triose phosphate isomerase, horseradish peroxidase, alkaline phosphatase, asparaginase, glucose oxidase, beta-galactosidase, ribonuclease, urease, catalase, glucose-6-phosphate dehydrogenase, glucoamylase and acetylcholinesterase. The detection can be accomplished by colometric methods which employ a chromogenic substrate for the enzyme. Detection can be accomplished by

colometric methods which employ a chromogenic substrate for the enzyme. Detection can also be accomplished by visual comparison of the extent of enzymatic reaction of a substrate with similarly prepared standards (see Harlow and Lane, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory 1988; Ausubel et al, eds., Current Protocols in Molecular Biology, Wiley Interscience, N.Y. 1987, 1992).

Detection may be accomplished using any of a variety of other immunoassays. For example, by radiolabeling of the antibodies or antibody fragments, it is possible to detect R-PTPase through the use of a radioimmunoassay (RIA). A good description of RIA may be found in Laboratory Techniques and Biochemistry in Molecular Biology, by Work et al, North Holland Publishing Company, NY (1978) with particular reference to the chapter entitled "An Introduction to Radioimmune Assay and Related Techniques" by Chard, incorporated entirely by reference herein. The radioactive isotope can be detected by such means as the use of a gamma-counter, a scintillation counter or by autoradiography.

It is also possible to label perlecan, perlecan fragments , the ~220 kDa HSPG, or ~220 kDa HSPG-fragments described herein, with a fluorescent compound. When the fluorescently labeled antibody is exposed to light of the proper wave length, its presence can then be detected due to fluorescence. Among the most commonly used fluorescent labeling compounds are fluorescein isothiocyanate, rhodamine, phycoerythrin, phycocyanin, allophycocyanin, o-phthaldehyde and fluorescamine, commercially available, e.g., from Molecular Probes, Inc. (Eugene, Oregon, U.S.A.).

The antibody can also be detectably labeled using fluorescence emitting metals such as ^{152}EU, or other of the lanthanide series. These metals can be attached to the antibody using such metal groups as diethylenetriamine pentaacetic acid (EDTA).

The antibody can also be detectably labeled by coupling it to a chemiluminescent compound. The presence of the chemiluminescent-tagged antibody is then determined by detecting the presence of luminescence that arises during the course of a chemical reaction, Examples of particularly useful chemiluminescent labeling compounds are luminol, isoluminol, theromatic acridinium ester, imidazole, acridinium salt, and oxalate ester.

Likewise, a bioluminescent compound may be used to label the antibody of the present invention. Bioluminescence is a type of chemiluminescence found in biological systems in which a catalytic protein increases the efficiency of the chemiluminescent reaction. The presence of a bioluminescent protein is determined by detecting the

presence of luminescence. Important bioluminescent compounds for purposes of labeling are luciferin, luciferase and aequorin.

The antibodies (or fragments thereof) useful in the present invention may be employed histologically, as in immunofluorescence or immunoelectron microscopy, for in situ detection of perlecan, perlecan fragments, the ~220 kDa HSPG or ~220 kDa HSPG fragments, of the present invention. In situ detection may be accomplished by removing a histological specimen from a patient, and providing the labeled antibody of the present invention to such a specimen. The antibody (or fragment) is preferably provided by applying or by overlaying the labeled antibody (or fragment) to a biological sample. Through the use of such a procedure, it is possible to determine not only the presence of perlecan, perlecan fragments, the ~220 kDa HSPG or ~220 kDa HSPG fragments, but also its distribution on the examined tissue. Using the present invention, those of ordinary skill will readily perceive that any of a wide variety of histological methods (such as staining procedures) can be modified in order to achieve such in situ detection.

In accordance with yet a further aspect of the present invention there are provided antibodies against perlecan, perlecan fragments, the ~220 kDa HSPG or a ~220 kDa HSPG-fragments. These antibodies can be used for a number of important diagnostic and/or therapeutic applications as described herein. In one aspect of the invention, polyclonal and/or monoclonal antibodies made against perlecan, perlecan fragments, the ~220 kDa HSPG or ~220 kDa HSPG fragments may be used for Western blot analysis (using standard Western blotting techniques knowledgeable to those skilled in the art) to detect the presence of amyloid protein-binding perlecan fragments or amyloid protein-binding ~220 kDa HSPGs in human tissues and in tissues of other species. Western blot analysis can also be used to determine the apparent size of each amyloid protein-binding perlecan or amyloid protein-binding ~220 kDa HSPG fragments. In addition, Western blotting following by scanning densitometry (known to those skilled in the art) can be used to quantitate and compare levels of each of the perlecan or ~220 kDa HSPG fragments in tissue samples, biological fluids or biopsies obtained from individuals with specific diseases (such as the amyloid diseases) in comparison to tissue samples, biological fluids or biopsies obtained from normal individuals or controls. Biological fluids, include, but are not limited to, blood, plasma, serum, cerebrospinal fluid, sputum, saliva, urine and stool.

In yet another aspect of the invention, polyclonal and/or monoclonal antibodies made against perlecan, perlecan fragments, the ~220 kDa HSPG or ~220 kDa

HSPG-fragments can be used for immunoprecipitation studies (using standard immunoprecipitation techniques known to one skilled in the art) to detect perlecan, perlecan fragments, the ~220 kDa HSPG or ~220 kDa HSPG-fragments which bind Aß or other amyloid proteins, in tissues, cells and/or biological fluids. Use of the perlecan, perlecan fragments, the ~220 kDa HSPG or ~220 kDa HSPG-fragment antibodies for immunoprecipitation studies can also be quantitated to determine relative levels of perlecan, perlecan fragments, the ~220 kDa HSPG or ~220 kDa HSPG-fragments which interact with Aß or other amyloid proteins, in tissues, cells and/or biological fluids. Quantitative immunoprecipitation can be used to compare levels of perlecan, perlecan fragments, the ~220 kDa HSPG or ~220 kDa HSPG-fragments in tissue samples, biological fluids or biopsies obtained from individuals with specific diseases (such as the amyloid diseases) in comparison to tissue samples, biological fluids or biopsies obtained from normal individuals or controls.

Diagnostic Applications

Use of Perlecan, ~220 kDa HSPG and/or Antibodies

Another aspect of the invention is to provide polyclonal and/or monoclonal antibodies to perlecan, perlecan fragments, the ~220 kDa HSPG or ~220 kDa HSPG-fragments which would be used to specifically detect perlecan, perlecan fragments, the ~220 kDa HSPG or ~220 kDa HSPG-fragments in human tissues and/or biological fluids. In one preferred embodiment, polyclonal or monoclonal antibodies made against clean perlecan, perlecan fragments, the ~220 kDa HSPG or ~220 kDa HSPG-fragments, can be used to detect and quantify perlecan, perlecan fragments, the ~220 kDa HSPG or ~220 kDa HSPG-fragments in human tissues and/or biological fluids. For detection of perlecan, perlecan fragments, the ~220 kDa HSPG or ~220 kDa HSPG-fragments in human tissues, cells, and/or in cell culture, the polyclonal and/or monoclonal antibodies can be used using standard immunohistochemical and immunocytochemical techniques, knowledgeable to one skilled in the art.

For detection and quantitation of perlecan, perlecan fragments, the ~220 kDa HSPG or ~220 kDa HSPG-fragments in biological fluids, including plasma, serum, urine, cerebrospinal fluid, sputum, and/or stool, various types of ELISA assays can be used, knowledgeable to one skilled in the art. In a preferred embodiment, a sandwich type of ELISA can be used. Using this preferred method a pilot study is first implemented to determine the quantity of binding of a clean perlecan, perlecan fragment, ~220 kDa HSPG or ~220 kDa HSPG-fragment monoclonal antibody to

microtiter wells. Once this is determined, aliquots (usually in 40 µl of TBS; pH 7.4) of the antibody are allowed to bind overnight to microtiter wells (Maxisorb C plate from Nunc) at 4°C. A series of blank wells not containing any primary perlecan, perlecan fragment, ~220 kDa HSPG or ~220 kDa HSPG-fragment specific monoclonal antibody are also used as controls. The next day, non-bound monoclonal antibody is shaken off the microtiter wells. All of the microtiter wells (including the blank wells) are then blocked by incubating for 2 hours with 300 µl of Tris-buffered saline containing 0.05% Tween-20 (TTBS) plus 2% bovine serum albumin, followed by 5 rinses with TTBS. 200 µl of plasma, serum, urine, cerebrospinal fluid, sputum, and/or stool and/or any other type of biological sample is then diluted (to be determined empirically) in TTBS containing 2% bovine serum albumin and placed in wells (in triplicate) containing bound perlecan, perlecan fragment, ~220 kDa HSPG or ~220 kDa HSPG-fragment antibody (or blank) and incubated for 2 hours at room temperature. The wells are then washed 5 times with TTBS. A second biotinylated-polyclonal antibody against perlecan, perlecan fragments, the ~220 kDa HSPG or ~220 kDa HSPG-fragments is then added to each well (usually in 40 µl of TBS; pH 7.4) and allowed to bind for 2 hours at room temperature to any perlecan, perlecan fragments, ~220 kDa HSPG or ~220 kDa HSPG-fragments captured by the first antibody. Following incubation, the wells are washed 5 times with TTBS. Bound materials are then detected by incubating with 100 µl of peroxidase-avidin complex (1:250 dilution in TTBS with 0.1% BSA) for 1 hour on a rotary shaker. After 5 washes with TTBS, a substrate solution (100 µl, OPD-Sigma Fast from Sigma Chemical Co., St. Louis, MO, USA) is added and allowed to develop significant color (usually 8-10 minutes). The reaction is stopped with 50 µl of 4N sulfuric acid and read on a standard spectrophotometer at 490 nm. This ELISA can be used to determine differences in perlecan, perlecan fragments, the ~220 kDa HSPG or ~220 kDa HSPG-fragments levels in biological fluids which can serve as diagnostic markers to follow the progression on a live patient during the progression of disease (*i.e.* monitoring of amyloid disease as an example). In addition, quantitative changes in perlecan, perlecan fragment, ~220 kDa HSPG or ~220 kDa HSPG-fragments levels can also serve as prognostic indicators monitoring how a live patient will respond to treatment which targets a given amyloid disease.

A competition assay may also be employed wherein antibodies specific to clean perlecan, perlecan fragments, the ~220 kDa HSPG or ~220 kDa HSPG-fragments are attached to a solid support and labeled perlecan, perlecan fragments, the ~220 kDa HSPG or ~220 kDa HSPG-fragments and a sample derived from a host are passed over

the solid support and the amount of label detected attached to the solid support can be correlated to the quantity of perlecan, perlecan fragments, the ~220 kDa HSPG or ~220 kDa HSPG-fragments in the sample, all as otherwise is known to those skilled in the art.

Another object of the present invention is to use clean perlecan or fragments thereof, in conjunction with perlecan specific antibodies, in an ELISA assay to detect potential perlecan autoantibodies in human biological fluids. In a preferred embodiment, perlecan will be used to initially bind to microtiter wells in an ELISA plate. A pilot study is first implemented to determine the quantity of binding of perlecan to microtiter wells. Once this is determined, aliquots (usually 1-2μg in 40 _l of TBS; pH 7.4) of perlecan or fragments thereof are allowed to bind overnight to microtiter wells (Maxisorb C plate from Nunc) at 4°C. All the microtiter wells (including blank wells without perlecan) are blocked by incubating for 2 hours with 300 μl of Tris-buffered saline (pH 7.4) with 0.05% Tween-20 (TTBS), containing 2% albumin. This is followed by 5 rinses with TTBS. The patient's biological fluids (ie, plasma, serum, cerebrospinal fluid, sputum, urine, and/or stool) are then used and 200 μl are diluted (to be determined empirically) with TTBS containing 2% bovine serum albumin, and placed in microtiter wells (in triplicate) containing perlecan or blank wells (which do not contain perlecan), and are incubated at 1.5 hours at room temperature. Any autoantibodies present in the biological fluids against perlecan will bind to the substrate bound perlecan (or fragments thereof). The wells are then rinsed by washing 5 times with TTBS. 100 μl of biotinylated polyclonal goat anti-human IgGs (Sigma Chemical company, St. Louis, MO, USA), diluted 1:500 in TTBS with 0.1% bovine serum albumin, is then aliquoted into each well. Bound materials are detected by incubating with 100 μl of peroxidase-avidin complex (1:250 dilution in TTBS with 0.1% bovine serum albumin) for 1 hour on a rotary shaker. Following 5 washes with TTBS, substrate solution (100 μl, OPD-Sigma Fast from Sigma Chemical Company, St. Louis, MO, USA) is added and allowed to develop significant color (usually 8-10 minutes). The reaction is stopped with 50 μl of 4N sulfuric acid added to each well and read on a standard spectrophotometer at 490 nm. This assay system can be used to not only detect the presence of autoantibodies against perlecan in biological fluids, but also to monitor the progression of disease by following elevation or diminution of perlecan autoantibody levels. It is believed that patients demonstrating excessive perlecan formation, deposition, accumulation and/or persistence as observed in the amyloid diseases, will also carry autoantibodies against perlecan in their biological fluids.

Various ELISA assay systems, knowledgeable to those skilled in the art, can be used to accurately monitor the degree of perlecan in biological fluids as a potential diagnostic indicator and prognostic marker for patients during the progression of disease (*i.e.* monitoring of an amyloid disease for example). In addition, quantitative changes in perlecan autoantibody levels can also serve as a prognostic indicator monitoring how a live patient will respond to treatment which targets a given amyloid disease.

Other diagnostic methods utilizing the invention include diagnostic assays for measuring altered levels of perlecan, perlecan fragments, the ~220 kDa HSPG or ~220 kDa HSPG-fragments in various tissues compared to normal control tissue samples. Assays used to detect levels of perlecan, perlecan fragments, the ~220 kDa HSPG or ~220 kDa HSPG-fragments in a sample derived from a host are otherwise well-known to those skilled in the art and included radioimmunoassays, competitive-binding assays, Western blot analysis and preferably ELISA assays (as described above).

Yet another aspect of the present invention is to use the antibodies recognizing perlecan, perlecan fragments, the ~220 kDa HSPG or ~220 kDa HSPG-fragments for labelings, for example, with a radionucleotide, for radioimaging or radioguided surgery, for in vivo diagnosis, and/or for in vitro diagnosis. In one preferred embodiment, radiolabeled antibodies made (by one skilled in the art) against perlecan, perlecan fragments, the ~220 kDa HSPG or ~220 kDa HSPG-fragments may be used as minimally invasive techniques to locate perlecan, perlecan fragments, the ~220 kDa HSPG or ~220 kDa HSPG-fragments and concurrent amyloid deposits in a living patient. These same imaging techniques could then be used at regular intervals (*i.e.* every 6 months) to monitor the progression of the amyloid disease by following the specific levels of perlecan, perlecan fragments, the ~220 kDa HSPG or ~220 kDa HSPG-fragments.

Therapeutic Applications

Use of Perlecan, ~220 kDa HSPG and/or Antibodies

Yet another aspect of the present invention is to make use of clean perlecan, perlecan fragments, the ~220 kDa HSPG or ~220 kDa HSPG-fragments thereof. The perlecan, perlecan fragments, ~220 kDa HSPG or ~220 kDa HSPG-fragments thereof can be used as potential blocking therapeutics for the interaction of perlecan, perlecan fragments, the ~220 kDa HSPG or ~220 kDa HSPG-fragments in a number of biological processes and diseases (such as in the amyloid diseases described above). In a preferred embodiment, perlecan, perlecan fragments, the ~220 kDa HSPG or ~220 kDa HSPG-fragments thereof may be used to block the interaction of perlecan,

perlecan fragments, the ~220 kDa HSPG or ~220 kDa HSPG-fragments with a given target (*i.e.* amyloid deposits). Inhibition by clean perlecan, perlecan fragments, the ~220 kDa HSPG or ~220 kDa HSPG-fragments may alleviate the continued amyloid formation, deposition, accumulation and/or persistence observed in a given patient. Likewise, in another preferred embodiment antibodies made against perlecan, perlecan fragments, the ~220 kDa HSPG or ~220 kDa HSPG-fragments thereof (as described above) may be given to a human patient as potential blocking antibodies to disrupt continued amyloid formation, deposition, accumulation and/or persistence in the given patient.

Preparations of clean perlecan, perlecan fragments, the ~220 kDa HSPG or ~220 kDa HSPG-fragments for parenteral administration include sterile aqueous or non-aqueous solutions, suspensions, and emulsions, which may contain axillary agents or excipients which are known in the art. Pharmaceutical compositions such as tablets, pills, tablets, caplets, soft and hard gelatin capsules, lozenges, sachets, cachets, vegicaps, liquid drops, elixers, suspensions, emulsions, solutions, syrups, tea bags, aerosols (as a solid or in a liquid medium), suppositories, sterile injectable solutions, sterile packaged powders, can be prepared according to routine methods and are known in the art.

In yet another aspect of the invention, clean perlecan, perlecan fragments, the ~220 kDa HSPG or ~220 kDa HSPG-fragments may be used as an effective therapy to block amyloid formation, deposition, accumulation and/or persistence as observed in the amyloid diseases. For example, the invention includes a pharmaceutical composition for use in the treatment of amyloidoses comprising a pharmaceutically effective amount of a clean perlecan, perlecan fragment, ~220 kDa HSPG or ~220 kDa HSPG-fragment anti-idiotypic antibody and a pharmaceutically acceptable carrier. The compositions may contain the perlecan, perlecan fragment, ~220 kDa HSPG or ~220 kDa HSPG-fragment anti-idiotypic antibody, either unmodified, conjugated to a potentially therapeutic compound, conjugated to a second protein or protein portion or in a recombinant form (*i.e.* chimeric or bispecific perlecan, perlecan fragment, ~220 kDa HSPG or ~220 kDa HSPG-fragment antibody). The compositions may additionally include other antibodies or conjugates. The antibody compositions of the invention can be administered using conventional modes of administration including, but not limited to, topical, intravenous, intra-arterial, intraperitoneal, oral, intralymphatic, intramuscular or intralumbar. Intravenous administration is preferred. The compositions of the invention can be a variety of dosage forms, with the preferred form

depending upon the mode of administration and the therapeutic application. Optimal dosage and modes of administration for an individual patient can readily be determined by conventional protocols.

Perlecan, perlecan fragments, the ~220 kDa HSPG or ~220 kDa HSPG-fragments, or antibodies of the present invention may be administered by any means that achieve their intended purpose, for example, to treat perlecan or ~220 kDa HSPG involved pathologies, such as Alzheimer's disease and other amyloid diseases, or other related pathologies, using a perlecan-derived or ~220 kDa HSPG-derived fragments as described herein, in the form of a pharmaceutical composition.

For example, administration of such a composition may be by various parenteral routes such as subcutaneous, intravenous, intradermal, intramuscular, intraperitoneal, intranasal, transdermal or buccal routes. Alternatively, or concurrently, administration may be by the oral route. Parenteral administration can be by bolus injection or by gradual perfusion over time.

A preferred mode of using a perlecan, perlecan fragment, ~220 kDa HSPG or ~220 kDa HSPG-fragment, or antibody pharmaceutical composition of the present invention is by oral administration or intravenous application.

A typical regimen for preventing, suppressing or treating perlecan-involved pathologies, such as Alzheimer's disease amyloidosis, comprises administration of an effective amount of clean perlecan, perlecan fragment, ~220 kDa HSPG or ~220 kDa HSPG-fragment-derived polypeptides, administered over a period of one or several days, up to and including between one week and about 24 months.

It is understood that the dosage of the clean perlecan, perlecan fragment, ~220 kDa HSPG or ~220 kDa HSPG-fragment of the present invention administered *in vivo* or *in vitro* will be dependent upon the age, sex, health, and weight of the recipient, kind of concurrent treatment, if any, frequency of treatment, and the nature of the effect desired. The most preferred dosage will be tailored to the individual subject, as is understood and determinable by one of skill in the art, without undue experimentation.

The total dose required for each treatment may be administered by multiple doses or in a single dose. A clean perlecan, perlecan fragment, ~220 kDa HSPG or ~220 kDa HSPG-fragment may be administered alone or in conjunction with other therapeutics directed to perlecan or ~220 kDa HSPG-involved pathologies, such as Alzheimer's disease or amyloid diseases, as described herein.

Effective amounts of a clean perlecan, perlecan fragment, ~220 kDa HSPG or ~220 kDa HSPG-fragment or composition, which may also include a perlecan, perlecan

fragment,~220 kDa HSPG or ~220 kDa HSPG-fragment derived antibody, are about 0.01μg to about 100mg/kg body weight, and preferably from about 10 μg to about 50 mg/kg body weight, such as 0.05, 0.07, 0.09, 0.1, 0.5, 0.7, 0.9., 1, 2, 5, 10, 20, 25, 30, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95 or 100 mg/kg.

Preparations for parenteral administration include sterile aqueous or non-aqueous solutions, suspensions, and emulsions, which may contain axillary agents or excipients which are known in the art. Pharmaceutical compositions comprising at least one perlecan, perlecan fragment, ~220 kDa HSPG or ~220 kDa HSPG-fragment, such as 1-10 or 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 perlecan, perlecan fragments, ~220 kDa HSPG or ~220 kDa HSPG-fragments, of the present invention may include all compositions wherein the perlecan, perlecan fragments,~220 kDa HSPG or ~220 kDa HSPG-fragments is contained in an amount effective to achieve its intended purpose. In addition to at least one clean perlecan, perlecan fragment, ~220 kDa HSPG or ~220 kDa HSPG-fragments, a pharmaceutical composition may contain suitable pharmaceutically acceptable carriers, such as excipients, carriers and/or axillaries which facilitate processing of the active compounds into preparations which can be used pharmaceutically.

Pharmaceutical compositions comprising at least one clean perlecan, perlecan fragment, ~220 kDa HSPG or ~220 kDa HSPG-fragment or antibody may also include suitable solutions for administration intravenously, subcutaneously, dermally, orally, mucosally, rectally or may by injection or orally, and contain from about 0.01 to 99 percent, preferably about 20 to 75 percent of active component (*i.e.* polypeptide or antibody) together with the excipient. Pharmaceutical compositions for oral administration include pills, tablets, caplets, soft and hard gelatin capsules, lozenges, sachets, cachets, vegicaps, liquid drops, elixers, suspensions, emulsions, solutions, and syrups.

The clean perlecan, perlecan fragment, ~220 kDa HSPG or ~220 kDa HSPG-fragment for Alzheimer's disease and other central nervous system amyloidoses may be optimized to cross the blood-brain barrier. Methods of introductions include but are not limited to systemic administration, parenteral administration, *i.e.* via an intraperitoneal, intravenous, perioral, subcutaneous, intramuscular, intraarterial, intradermal, intramuscular, intranasal, epidural and oral routes. In a preferred embodiment, perlecan, perlecan fragments, the ~220 kDa HSPG or ~220 kDa HSPG-fragments may be directly administered to the cerebrospinal fluid by intraventricular injection. In a specific embodiment, it may be desirable to administer

perlecan, perlecan fragments, the ~220 kDa HSPG or ~220 kDa HSPG-fragments locally to the area or tissue in need of treatment; this may be achieved by, for example, and not by way of limitation, local infusion during surgery, topical application, by injection, by infusion using a cannulae with osmotic pump, by means of a catheter, by means of a suppository, or by means of an implant.

In yet another embodiment, clean perlecan, perlecan fragments, the ~220 kDa HSPG or ~220 kDa HSPG-fragments may be delivered in a controlled release system, such as an osmotic pump. In yet another embodiment, a controlled release system can be placed in proximity to the therapeutic target, ie. the brain, thus requiring only a fraction of the systemic dose.

In yet another aspect of the present invention, peptidomimetic compounds modeled from clean perlecan, perlecan fragments, the ~220 kDa HSPG or ~220 kDa HSPG-fragments identified as binding Aß or other amyloid proteins, may serve as potent inhibitors of amyloid formation, deposition, accumulation and/or persistence in Alzheimer's disease and other amyloidoses. Peptidomimetic modeling is implemented by standard procedures known to those skilled in the art.

Recombinant DNA technology, including human gene therapy, has direct applicability to perlecan, perlecan fragments, the ~220 kDa HSPG or ~220 kDa HSPG-fragments, of this invention. One skilled in the art can take peptide sequences from these molecules and create corresponding nucleotide sequences that code for the corresponding peptide sequences. These sequences can be cloned into vectors such as retroviral vectors, and the like. These vectors can, in turn, be transfected into human cells such as hepatocytes or fibroblasts, and the like. Such transfected cells can be introduced into humans to treat amyloid diseases. Alternatively, the genes can be introduced into the patients directly. The basic techniques of recombinant DNA technology are known to those of ordinary skill in the art and are disclosed in Recombinant DNA Second Edition, Watson, et al., W.H. Freeman and Company, New York, 1992, which is hereby incorporated by reference.

In yet another aspect of the present invention is to use anti-idiotypic antibodies to perlecan, perlecan fragments, the ~220 kDa HSPG or ~220 kDa HSPG-fragments as potent inhibitors of amyloid formation, deposition, accumulation and/or persistence in Alzheimer's disease and other amyloidoses. Production of anti-idiotypic antibodies is implemented by standard procedures knowledgeable to those skilled in the art.

Use of the Perlecan Product for Production of New Animal Models

Infusion Models for Alzheimer's Disease and Down's Syndrome Amyloidosis

The production of "clean" perlecan or the ~220 kDa HSPG product as described in the present invention can also be used to produce new animal models of the amyloidoses. For example, as a new model of Alzheimer's disease amyloidosis, perlecan, perlecan fragments, the ~220 kDa HSPG or ~220 kDa HSPG-fragments can be continuously infused in combination with beta-amyloid protein (Aß) into the hippocampus of groups of rats or mice (as described in detail in section 7.1.1 and 7.1.2. In a preferred embodiment perlecan, perlecan fragments, the ~220 kDa HSPG or ~220 kDa HSPG-fragments (25 µg) is dissolved in water in a microcentrifuge tube containing 50 µg of Aß (1-40) or (1-42). Using the described methods of Snow et al (Neuron 12:219-234, 1994) herewith incorporated by reference, perlecan, perlecan fragments, the ~220 kDa HSPG or ~220 kDa HSPG-fragments plus Aß is continuously infused for 1 week into hippocampus (via stereotaxic surgeries knowledgeable by one skilled in the art) of groups (usually 10) of 3 month old Sprague-Dawley rats. Following the 1 week infusion the animals are sacrificed and the brains are removed as described in Snow et al (Neuron 12:219-234, 1994), and 6-8 µm serial sections spanning through the entire infusion site are cut from paraffin embedded blocks or from frozen sections. The extent of amyloid deposition per animal is then detected by Congo red staining (as viewed under polarized light) or Thioflavin S fluorescence and quantitated in a blind study using an arbitrary scoring method as described by Snow et al (Neuron 12:219-234, 1994). The use of perlecan, perlecan fragments, the ~220 kDa HSPG or ~220 kDa HSPG-fragments in this model can be used as a rapid model of fibrillar Aß amyloid deposition, accumulation and persistence in vivo. In addition, this model may be used to rapidly screen potential therapeutics targeting fibrillar Aß amyloid formation, deposition, accumulation and/or persistence. In a preferred embodiment, perlecan, perlecan fragments, the ~220 kDa HSPG or ~220 kDa HSPG-fragments plus Aß plus therapeutic compound is directly infused into the hippocampus (as described above) of a group of animals and comparisons are made to a group of animals infused with only perlecan, perlecan fragments, the ~220 kDa HSPG or ~220 kDa HSPG-fragments plus Aß. Compounds or drugs found to reduce amyloid formation, deposition, accumulation and/or persistence (as determined by Congo red or Thioflavin S scoring) *in vivo* are then identified as having potential therapeutic value.

In another preferred embodiment, the potentially therapeutic compound can be tested to reduce amyloid persistence over prolonged periods of time. In this model, groups of animals (usually 10 animals per group) are infused with perlecan, perlecan fragments, the ~220 kDa HSPG or ~220 kDa HSPG-fragments plus Aß plus

therapeutic compound and directly compared to groups of animals (usually 10 animals per group) infused with perlecan, perlecan fragments, the ~220 kDa HSPG or ~220 kDa HSPG-fragments plus Aß. Following a 1 week infusion (as described above), the cannulae are removed with the animals under anesthesia, and the animals are then allowed to recover until sacrifice 1, 3, 6 or 12 months later. Serial sections are cut and amyloid is scored as described above. It is believed that persistent amyloid deposits can be observed in animals infused with perlecan, perlecan fragments, the ~220 kDa HSPG or ~220 kDa HSPG-fragments plus Aß. Potent therapeutic compounds will be those that effectively reduce the amount of amyloid observed in comparison to those animals not given the therapeutic compound. These compounds can therefore be referred to as compounds which effectively reduce amyloid persistence in vivo.

In yet another preferred embodiment, potentially therapeutic compounds can be tested for reducing or eliminating pre-formed amyloid deposits. In this model, two groups of animals (usually 10 animals per group) are infused with perlecan, perlecan fragments, the ~220 kDa HSPG or ~220 kDa HSPG-fragments plus Aß. Following a 1 week infusion (as described above), the cannulae and osmotic pumps are changed (with the animals under anesthesia), and a new cannulae connected by vinyl tubing to a new osmotic pump, contains either vehicle only (*i.e.* double distilled water) or the potential therapeutic compound. Following a 1 week continuous infusion of either the vehicle or the potential therapeutic compound of interest, the animals are sacrificed. Serial sections are then cut through the entire infusion site and the extent of amyloid is measured by arbitrary blind scoring as described above. Potent therapeutic compounds will be those that are able to effectively remove pre-formed amyloid deposits. It is anticipated that little to no reduction in the amount of amyloid will be observed in the group of animals infused with vehicle only. These compounds can therefore be referred to as therapeutic compounds which effectively reduce pre-formed amyloid deposits in vivo.

New Animal Models of AA Amyloidosis

The consistent and reliable production of "clean" perlecan or the ~220 kDa HSPG as described in the present invention can also be used to produce a new animal model of AA amyloidosis. For example, perlecan, perlecan fragments, the ~220 kDa HSPG or ~220 kDa HSPG-fragments can be continuously infused into systemic organs (*i.e.* kidney, liver, spleen, lung or heart) or injected daily into the tail veins of rats or mice, in combination with AA amyloid protein. In a preferred embodiment perlecan, perlecan fragments, the ~220 kDa HSPG or ~220 kDa HSPG-fragments are dissolved

in water in a microcentrifuge tube containing AA amyloid protein. Using the described methods of Snow et al (Neuron 12:219-234, 1994), perlecan, perlecan fragments, the ~220 kDa HSPG or ~220 kDa HSPG-fragments plus AA amyloid is continuously infused for 1 week into a systemic organ of choice (via stereotaxic surgeries knowledgeable by one skilled in the art) of groups (usually 10) of adult rats or mice. Alternatively, AA amyloid protein + /- perlecan, perlecan fragments, the ~220 kDa HSPG or ~220 kDa HSPG-fragments are injected daily into the tail veins of a group of rats or mice. Following the 1 week experimental period, the animals are sacrificed and the systemic organs are removed, and 6-8 μm serial sections are cut from paraffin embedded blocks or from frozen sections containing the tissues of interest. The extent of amyloid deposition in each tissue per animal is then detected by Congo red staining (as viewed under polarized light) or Thioflavin S fluorescence and quantitated in a blind study using an arbitrary scoring method as described by Snow et al (Neuron 12:219-234, 1994). The use of perlecan, perlecan fragments, the ~220 kDa HSPG or ~220 kDa HSPG-fragments in this model can be used as a rapid model of fibrillar AA amyloid deposition, accumulation and persistence in vivo. In addition, this model may be used to rapidly screen potential therapeutics targeting AA amyloid formation, deposition, accumulation and/or persistence. In a preferred embodiment, perlecan, perlecan fragments, the ~220 kDa HSPG or ~220 kDa HSPG-fragments plus AA amyloid plus therapeutic compound is directly infused or injected (as described above) into a group of animals and comparisons are made to a group of animals infused or injected with only perlecan, perlecan fragments, the ~220 kDa HSPG or ~220 kDa HSPG-fragments plus Aß. Compounds or drugs found to reduce amyloid formation, deposition, accumulation and/or persistence (as determined by Congo red or Thioflavin S scoring) in vivo are then identified as having potential therapeutic value.

New Animal Models of AL Amyloidosis

The consistent and reliable production of "clean" perlecan or the ~220 kDa HSPG as described in the present invention can also be used to produce a new animal model of AL amyloidosis. For example, perlecan, perlecan fragments, the ~220 kDa HSPG or ~220 kDa HSPG-fragments can be continuously infused into systemic organs (*i.e.* kidney, liver, spleen, lung or heart) or injected daily into the tail veins of rats or mice, in combination with AL amyloid protein. In a preferred embodiment perlecan, perlecan fragments, the ~220 kDa HSPG or ~220 kDa HSPG-fragments are dissolved in water in a microcentrifuge tube containing AL amyloid protein. Using the described methods of Snow et al (Neuron 12:219-234, 1994), perlecan, perlecan fragments, the

~220 kDa HSPG or ~220 kDa HSPG-fragments plus AL amyloid is continuously infused for 1 week into a systemic organ of choice (via stereotaxic surgeries knowledgeable by one skilled in the art) of groups (usually 10) of adult rats or mice. Alternatively, AL amyloid protein + /- perlecan, perlecan fragments, the ~220 kDa HSPG or ~220 kDa HSPG-fragments are injected daily into the tail veins of a group of rats or mice. Following the 1 week experimental period, the animals are sacrificed and the systemic organs are removed, and 6-8 μm serial sections are cut from paraffin embedded blocks or from frozen sections containing the tissues of interest. The extent of amyloid deposition in each tissue per animal is then detected by Congo red staining (as viewed under polarized light) or Thioflavin S fluorescence and quantitated in a blind study using an arbitrary scoring method as described by Snow et al (Neuron 12:219-234, 1994). The use of perlecan, perlecan fragments, the ~220 kDa HSPG or ~220 kDa HSPG-fragments in this model can be used as a rapid model of fibrillar AL amyloid deposition, accumulation and persistence in vivo. In addition, this model may be used to rapidly screen potential therapeutics targeting AL amyloid formation, deposition, accumulation and/or persistence. In a preferred embodiment, perlecan, perlecan fragments, the ~220 kDa HSPG or ~220 kDa HSPG-fragments plus AL amyloid plus therapeutic compound are directly infused or injected (as described above) into a group of animals and comparisons are made to a group of animals infused or injected with only perlecan, perlecan fragments, the ~220 kDa HSPG or ~220 kDa HSPG-fragments plus AL amyloid. Compounds or drugs found to reduce amyloid formation, deposition, accumulation and/or persistence (as determined by Congo red or Thioflavin S scoring) in vivo are then identified as having potential therapeutic value.

New Animal Models of Transthyretin/Prealbumin Amyloidosis

The consistent and reliable production of "clean" perlecan or the ~220 kDa HSPG as described in the present invention can also be used to produce a new animal model of transthyretin/prealbumin amyloidosis. For example, perlecan, perlecan fragments, the ~220 kDa HSPG or ~220 kDa HSPG-fragments can be continuously infused or injected daily into the sciatic nerve, dorsal root ganglion or autonomic ganglion of rats or mice, in combination with various normal or mutated transthyretin/ prealbumin proteins. In a preferred embodiment perlecan, perlecan fragments, the ~220 kDa HSPG or ~220 kDa HSPG-fragments are dissolved in water in a microcentrifuge tube containing of normal or mutated transthyretin/ prealbumin proteins. Using the described methods of Snow et al (Neuron 12:219-234, 1994), the

perlecan, perlecan fragments, the ~220 kDa HSPG or ~220 kDa HSPG-fragments plus normal or mutated transthyretin/ prealbumin amyloid are continuously infused for 1 week into sciatic nerve, dorsal root ganglion or autonomic ganglion (via stereotaxic surgeries knowledgeable by one skilled in the art) of groups (usually 10) of adult rats or mice. Alternatively, normal or mutated transthyretin/ prealbumin + /- perlecan, perlecan fragments, the ~220 kDa HSPG or ~220 kDa HSPG-fragments are injected daily into sciatic nerve, dorsal root ganglion or autonomic ganglion of a group of rats or mice. Following the 1 week experimental period, the animals are sacrificed and the pertinent tissues are removed, and 6-8 μm serial sections are cut from paraffin embedded blocks or from frozen sections containing the tissues of interest. The extent of amyloid deposition in each tissue per animal is then detected by Congo red staining (as viewed under polarized light) or Thioflavin S fluorescence and quantitated in a blind study using an arbitrary scoring method as described by Snow et al (Neuron 12:219-234, 1994). The use of perlecan, perlecan fragments, the ~220 kDa HSPG or ~220 kDa HSPG-fragments in this model can be used as a rapid model of fibrillar transthyretin/ prealbumin amyloid deposition, accumulation and persistence in vivo. In addition, this model may be used to rapidly screen potential therapeutics targeting transthyretin/ prealbumin amyloid formation, deposition, accumulation and/or persistence. In a preferred embodiment, perlecan, perlecan fragments, the ~220 kDa HSPG or ~220 kDa HSPG-fragments plus normal or mutated transthyretin/ prealbumin plus therapeutic compound are directly infused or injected (as described above) into a group of animals and comparisons are made to a group of animals infused or injected with only perlecan, perlecan fragments, the ~220 kDa HSPG or ~220 kDa HSPG-fragments plus normal or mutated transthyretin/ prealbumin. Compounds or drugs found to reduce amyloid formation, deposition, accumulation and/or persistence (as determined by Congo red or Thioflavin S scoring) in vivo are then identified as having potential therapeutic value.

New Animal Models of Beta$_2$-Microglobulin Amyloidosis

The consistent and reliable production of "clean" perlecan or the ~220 kDa HSPG as described in the present invention can also be used to produce a new animal model of beta$_2$-microglobulin amyloidosis. For example, perlecan, perlecan fragments, the ~220 kDa HSPG or ~220 kDa HSPG-fragments can be continuously infused into the bloodstream (for example, through external jugular vein to superior vena cava) or injected daily into the tendon or hind leg (adjacent to the medial nerve) of rats or mice, in combination with beta$_2$-microglobulin. In a preferred embodiment perlecan, perlecan

fragments, the ~220 kDa HSPG or ~220 kDa HSPG-fragments are dissolved in water in a microcentrifuge tube containing beta$_2$-microglobulin. Using the described methods of Snow et al (Neuron 12:219-234, 1994), the perlecan, perlecan fragments, the ~220 kDa HSPG or ~220 kDa HSPG-fragments plus beta$_2$-microglobulin are continuously infused for 1 week into the bloodstream (via stereotaxic surgeries knowledgeable by one skilled in the art) of groups (usually 10) of adult rats or mice. Alternatively, beta$_2$-microglobulin + /- perlecan, perlecan fragments, the ~220 kDa HSPG or ~220 kDa HSPG-fragments are injected daily into the tendon or hind leg of a group of rats or mice. Following the 1 week experimental period, the animals are sacrificed and the pertinent tissues are removed, and 6-8 μm serial sections are cut from paraffin embedded blocks or from frozen sections containing the tissues of interest. The extent of amyloid deposition in each tissue per animal is then detected by Congo red staining (as viewed under polarized light) or Thioflavin S fluorescence and quantitated in a blind study using an arbitrary scoring method as described by Snow et al (Neuron 12:219-234, 1994). The use of perlecan, perlecan fragments, the ~220 kDa HSPG or ~220 kDa HSPG-fragments in this model can be used as a rapid model of beta$_2$-microglobulin amyloid deposition, accumulation and persistence in vivo. In addition, this model may be used to rapidly screen potential therapeutics targeting beta$_2$-microglobulin amyloid formation, deposition, accumulation and/or persistence. In a preferred embodiment, perlecan, perlecan fragments, the ~220 kDa HSPG or ~220 kDa HSPG-fragments plus beta$_2$-microglobulin plus therapeutic compound is directly infused or injected (as described above) into a group of animals and comparisons are made to a group of animals infused or injected with only perlecan, perlecan fragments, the ~220 kDa HSPG or ~220 kDa HSPG-fragments plus beta$_2$-microglobulin. Compounds or drugs found to reduce amyloid formation, deposition, accumulation and/or persistence (as determined by Congo red or Thioflavin S scoring) in vivo are then identified as having potential therapeutic value.

New Animal Models of Amylin (Islet Amyloid Polypeptide) Amyloidosis

The consistent and reliable production of "clean" perlecan or the ~220 kDa HSPG as described in the present invention can also be used to produce a new animal model of amylin (islet amyloid polypeptide) amyloidosis. For example, perlecan, perlecan fragments, the ~220 kDa HSPG or ~220 kDa HSPG-fragments can be continuously infused or daily injected into the pancreas or bloodstream of rats or mice, in combination with human amylin. In a preferred embodiment perlecan, perlecan fragments, the ~220 kDa HSPG or ~220 kDa HSPG-fragments are dissolved in water

in a microcentrifuge tube containing amylin. Using the described methods of Snow et al (Neuron 12:219-234, 1994), perlecan, perlecan fragments, the ~220 kDa HSPG or ~220 kDa HSPG-fragments plus amylin are continuously infused or daily injected for 1 week into the pancreas or bloodstream (via stereotaxic surgeries knowledgeable by one skilled in the art) of groups (usually 10) of adult rats or mice. Following the 1 week experimental period, the animals are sacrificed and the pancreas is removed, and 6-8 μm serial sections are cut from paraffin embedded blocks or from frozen sections containing the pancreas. The extent of amyloid deposition in the pancreas per animal is then detected by Congo red staining (as viewed under polarized light) or Thioflavin S fluorescence and quantitated in a blind study using an arbitrary scoring method as described by Snow et al (Neuron 12:219-234, 1994). The use of perlecan, perlecan fragments, the ~220 kDa HSPG or ~220 kDa HSPG-fragments in this model can be used as a rapid model of amylin (islet amyloid) deposition, accumulation and persistence in vivo. In addition, this model may be used to rapidly screen potential therapeutics targeting amylin (islet amyloid) formation, deposition, accumulation and/or persistence. In a preferred embodiment, perlecan, perlecan fragments, the ~220 kDa HSPG or ~220 kDa HSPG-fragments plus amylin plus therapeutic compound is directly infused or injected (as described above) into a group of animals and comparisons are made to a group of animals infused or injected with only perlecan, perlecan fragments, the ~220 kDa HSPG or ~220 kDa HSPG-fragments plus amylin. Compounds or drugs found to reduce amyloid formation, deposition, accumulation and/or persistence (as determined by Congo red or Thioflavin S scoring) in vivo are then identified as having potential therapeutic value.

New Animal Models of Endocrine Type Amyloidosis

The consistent and reliable production of "clean" perlecan or the ~220 kDa HSPG as described in the present invention can also be used to produce a new animal model of endocrine amyloidosis, such as observed when a variant of calcitonin is found in the amyloid of medullary carcinoma of the thyroid, as well as in the islets of Langerhans in the pancreas of patients with type II (non-insulin dependent) diabetes. For example, perlecan, perlecan fragments, the ~220 kDa HSPG or ~220 kDa HSPG-fragments can be continuously infused or daily injected into the thyroid gland or pancreas of rats or mice, in combination with calcitonin. In a preferred embodiment perlecan, perlecan fragments, the ~220 kDa HSPG or ~220 kDa HSPG-fragments are dissolved in water in a microcentrifuge tube containing calcitonin. Using the described methods of Snow et al (Neuron 12:219-234, 1994), perlecan, perlecan fragments, the

~220 kDa HSPG or ~220 kDa HSPG-fragments plus calcitonin are continuously infused or daily injected for 1 week into the thyroid gland or pancreas (via stereotaxic surgeries knowledgeable by one skilled in the art) of groups (usually 10) of adult rats or mice. Following the 1 week experimental period, the animals are sacrificed and the thyroid gland or pancreas is removed, and 6-8 μm serial sections are cut from paraffin embedded blocks or from frozen sections containing the thyroid gland or pancreas. The extent of amyloid deposition in the thyroid gland or pancreas per animal is then detected by Congo red staining (as viewed under polarized light) or Thioflavin S fluorescence and quantitated in a blind study using an arbitrary scoring method as described by Snow et al (Neuron 12:219-234, 1994). The use of perlecan, perlecan fragments, the ~220 kDa HSPG or ~220 kDa HSPG-fragments in this model can be used as a rapid model of endocrine amyloid deposition, accumulation and persistence in vivo. In addition, this model may be used to rapidly screen potential therapeutics targeting endocrine amyloid formation, deposition, accumulation and/or persistence. In a preferred embodiment, perlecan, perlecan fragments, the ~220 kDa HSPG or ~220 kDa HSPG-fragments plus calcitonin plus therapeutic compound is directly infused or injected (as described above) into a group of animals and comparisons are made to a group of animals infused or injected with only perlecan, perlecan fragments, the ~220 kDa HSPG or ~220 kDa HSPG-fragments plus calcitonin. Compounds or drugs found to reduce amyloid formation, deposition, accumulation and/or persistence (as determined by Congo red or Thioflavin S scoring) in vivo are then identified as having potential therapeutic value.

New Animal Models of Prion Protein Amyloidosis

The consistent and reliable production of "clean" perlecan or the ~220 kDa HSPG as described in the present invention can also be used to produce a new animal model of prion protein (PrP) amyloidosis. For example, perlecan, perlecan fragments, the ~220 kDa HSPG or ~220 kDa HSPG-fragments can be continuously infused in combination with PrP protein into the hippocampus of groups of rats or mice. In a preferred embodiment perlecan, perlecan fragments, the ~220 kDa HSPG or ~220 kDa HSPG-fragments are dissolved in water in a microcentrifuge tube containing PrP 27-30. Using the described methods of Snow et al (Neuron 12:219-234, 1994), perlecan, perlecan fragments, the ~220 kDa HSPG or ~220 kDa HSPG-fragments plus PrP are continuously infused for 1 week into hippocampus (via stereotaxic surgeries knowledgeable by one skilled in the art) of groups (usually 10) of adult rats or mice. Following the 1 week infusion the animals are sacrificed and the brains are removed

as described in Snow et al (Neuron 12:219-234, 1994), and 6-8 μm serial sections spanning through the entire infusion site are cut from paraffin embedded blocks or from frozen sections. The extent of amyloid deposition per animal is then detected by Congo red staining (as viewed under polarized light) or Thioflavin S fluorescence and quantitated in a blind study using an arbitrary scoring method as described by Snow et al (Neuron 12:219-234, 1994). The use of perlecan, perlecan fragments, the ~220 kDa HSPG or ~220 kDa HSPG-fragments in this model can be used as a rapid model of PrP amyloid deposition, accumulation and persistence in vivo. In addition, this model may be used to rapidly screen potential therapeutics targeting PrP amyloid formation, deposition, accumulation and/or persistence. In a preferred embodiment, perlecan, perlecan fragments, the ~220 kDa HSPG or ~220 kDa HSPG-fragments plus PrP 27-30 plus therapeutic compound is directly infused into the hippocampus (as described above) of a group of animals and comparisons are made to a group of animals infused with only perlecan, perlecan fragments, the ~220 kDa HSPG or ~220 kDa HSPG-fragments plus PrP 27-30. Compounds or drugs found to reduce amyloid formation, deposition, accumulation and/or persistence (as determined by Congo red or Thioflavin S scoring) in vivo are then identified as having potential therapeutic value.

CLAIMS

We claim:

1. A method of preparation of substantially pure proteoglycan from an extract source, the method comprising one or more steps selected from the group of steps consisting of: a) isolation of an extracted proteoglycan by molecular sieve column chromatography, b) cation exchange column chromatography, c) anion exchange column chromatography, and d) chromatography using a column containing immobilized glycosaminoglycans.

2. The method of Claim 1 wherein the proteoglycan is perlecan.

3. The method of Claim 1 wherein the proteoglycan is ~220 kDa heparan sulfate proteoglycan.

4. The method of Claim 1 wherein the extract source is Engelbreth-Holm-Swarm tumor tissue.

5. The method of Claim 1 whereby the step of isolation by molecular sieve column chromatography employs a Sephacryl S-400 column.

6. The method of Claim 1 whereby the step of isolation by cation exchange column chromatography employs a Sulphopropyl Sepharose column.

7. The method of Claim 1 whereby the step of isolation by anion exchange column chromatography employs a DEAE-Sephacel column.

8. The method of Claim 1 whereby the step of isolation by a molecular sieve column employs a second column in the form of a Sephacryl S-1000 column.

9. The method of Claim 1 whereby the step of isolation by a column containing immobilized glycosaminoglycans employs a heparin-Sepharose column.

10. The method of Claim 1 wherein the step(s) of isolation proceed to a level of contaminating proteins, proteoglycans or macromolecules of less than 1%.

11. The method of Claim 1 wherein the step(s) of isolation proceed to a level of contamination by DNA of less than 1%.

12. The method of Claim 10 wherein the step(s) of isolation proceed to a level of contaminating proteins, proteoglycans or macromolecules of less than or equal to 0.1%.

13. A clean perlecan produced by the method of Claim 2.

14. The clean perlecan of Claim 13 having a level of contaminating proteins, proteoglycans, macromolecules or DNA of less than 1%.

15. A method of making an antibody, the method comprising producing antibodies from the clean perlecan of Claim 13.

16. The method of Claim 15 wherein the making of the antibody comprises production of at least one type of antibody selected from the group of antibody types consisting of polyclonal, monoclonal, and chimeric antibodies and anti-idiotypic antibodies.

17. A method to diagnose a disease or a susceptibility to a disease related to the levels of perlecan, perlecan-derived protein or glycosaminoglycans fragments, ~220 kDa HSPG, or ~220 kDa HSPG-derived protein or glycosaminoglycans fragments, the method comprising determining levels of perlecan, a particular perlecan-derived fragment, or the ~220 kDa HSPG, or ~220 kDa derived fragment in a sample, whereby the levels are indicative of the presence of a disease, susceptibility to a disease, or progression of said disease.

18. The method of Claim 17 wherein said disease is an amyloid disease.

19. The method of Claim 17 wherein the sample assayed is a biological fluid.

20. The method of Claim 15 further comprising radiolabeling the antibodies for radioimaging or *in vivo* diagnosis for detection of perlecan, perlecan-derived protein or glycosaminoglycans fragments, ~220 kDa HSPG, or ~220 kDa HSPG-derived protein or glycosaminoglycan fragments.

21. A method for detection and quantification of perlecan and perlecan-derived fragments in biological fluids comprising a) allowing a first clean perlecan or perlecan-derived fragment antibody to bind to microtiter wells for a sufficient time to allow said binding, b) adding a quantity of biological fluid to the microtiter wells, c) incubating the biological fluid for sufficient time to allow binding of any perlecan or perlecan-derived fragment in the biological fluid to the first antibody on the microtiter wells, d) adding a second labeled antibody to the microtiter wells wherein the second labeled antibody is against perlecan or perlecan-derived fragment, but which is against a different epitope than the first antibody, and allowing the second antibody to bind to any perlecan or perlecan-derived fragment captured by the first antibody, and e) detecting bound materials using an appropriate substrate or label.

22. A method for detection and quantification of perlecan autoantibodies in biological fluids comprising a) allowing clean perlecan or a fragment thereof to bind to microtiter wells for a sufficient time to allow said binding, b) adding a quantity of biological fluid to the microtiter wells, c) incubating the biological fluid for sufficient time to allow binding of any perlecan autoantibody in the biological fluid to the clean perlecan or a fragment thereof on the microtiter wells, d) adding a labeled antibody to the microtiter wells wherein the labeled antibody is against human immunoglobulins

and allowing the antibody to bind to any perlecan autoantibody captured by the perlecan or a fragment thereof, and e) detecting bound materials using an appropriate substrate or label.

23. The method of Claim 21 wherein said biological fluid is selected from the group of biological fluids consisting of blood, plasma, serum, cerebrospinal fluid, sputum, saliva, urine and stool.

24. A method for the treatment of a patient having an identified clinical need to interfere with the pathological effects of amyloid, the method comprising administrating to the patient a therapeutically effective amount of clean perlecan, or a protein or glycosaminoglycan fragment thereof.

25. The method of Claim 24 wherein said amyloid disease is Alzheimer's disease.

26. A method for producing an animal model of amyloid diseases comprising a) injecting or infusing clean perlecan, the ~220 kDa HSPG, or fragments thereof, in combination with the appropriate amyloid protein into a given tissue or organ of a non-human mammal, and b) allowing sufficient time for the amyloid protein plus perlecan or the ~220 kDa HSPG to be co-deposited in said tissue or organ, c) detecting the amyloid deposit in said organ in tissue using standard staining techniques for fibrillar amyloid.

27. The method of Claim 26 wherein the amyloid disease is Alzheimer's disease and the appropriate amyloid protein is the beta-amyloid protein (Aß).

28. The method of Claim 26 wherein the tissue or organ is brain tissue.

29. The method of Claim 26 wherein the non-human mammal is a rat.

30. An *in vivo* assay for selecting a candidate therapeutic for inhibiting congophilic and fibrillar amyloid deposition/persistence, comprising a) administering a candidate reagent to a first animal in a first infusate comprising an amyloid protein and clean perlecan or the ~220 kDa HSPG by continuous infusion at an infusion site into said tissue or organ, b) selecting the candidate reagent as a candidate therapeutic for inhibiting congophilic and fibrillar amyloid deposition/persistence if the first infusate diminishes Congo red and Thioflavin S staining indicative of fibrillar amyloid deposition/persistence at the infusion site, as compared with a second animal receiving a second infusate consisting essentially of the amyloid protein and perlecan or the ~220 kDa HSPG.

50 Grams EHS Tumor

↓

Multiple Extractions in 4 M Guanidine-HCl containing 0.5 % CHAPS and Protease Inhibitors

↓

Centrifugation at 17,000 x g for 30 min

↓

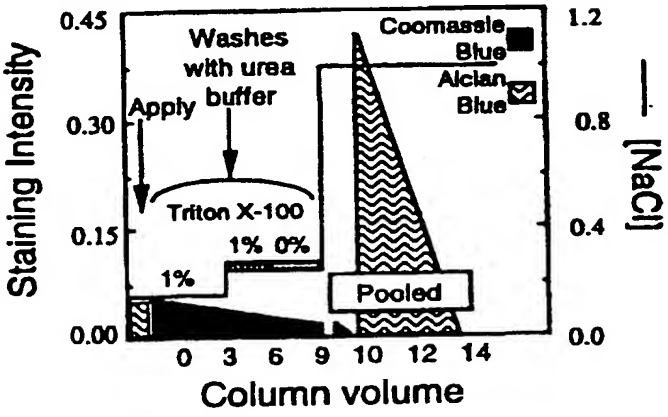
Collect Supernatants

↓

Apply to Sephacryl S-400 Column and Elute with 7 M Urea, 0.2 M NaCl, 0.1 % CHAPS, 50 mM Tris (pH 8.0) containing Protease Inhibitors

↓

Apply Void Volume Fractions to a 50 ml DEAE-Sephacel Column



↓

Apply PG Fractions to Sephacryl S-1000 Column and Elute with 1 M Urea, 50 mM Tris-HCl (pH 8.0) containing Protease Inhibitors

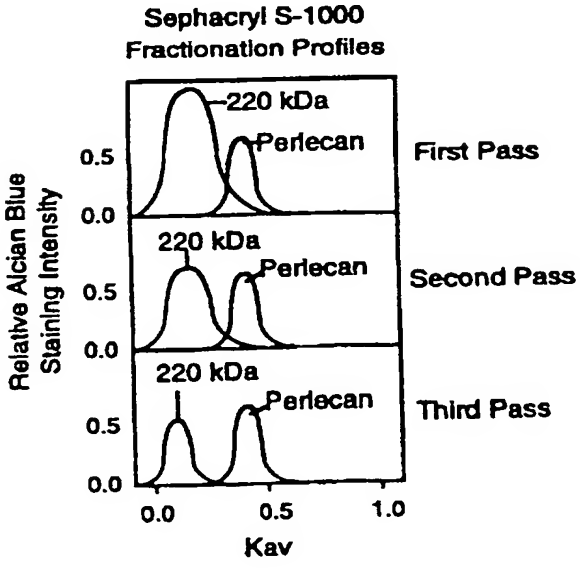


FIGURE 1

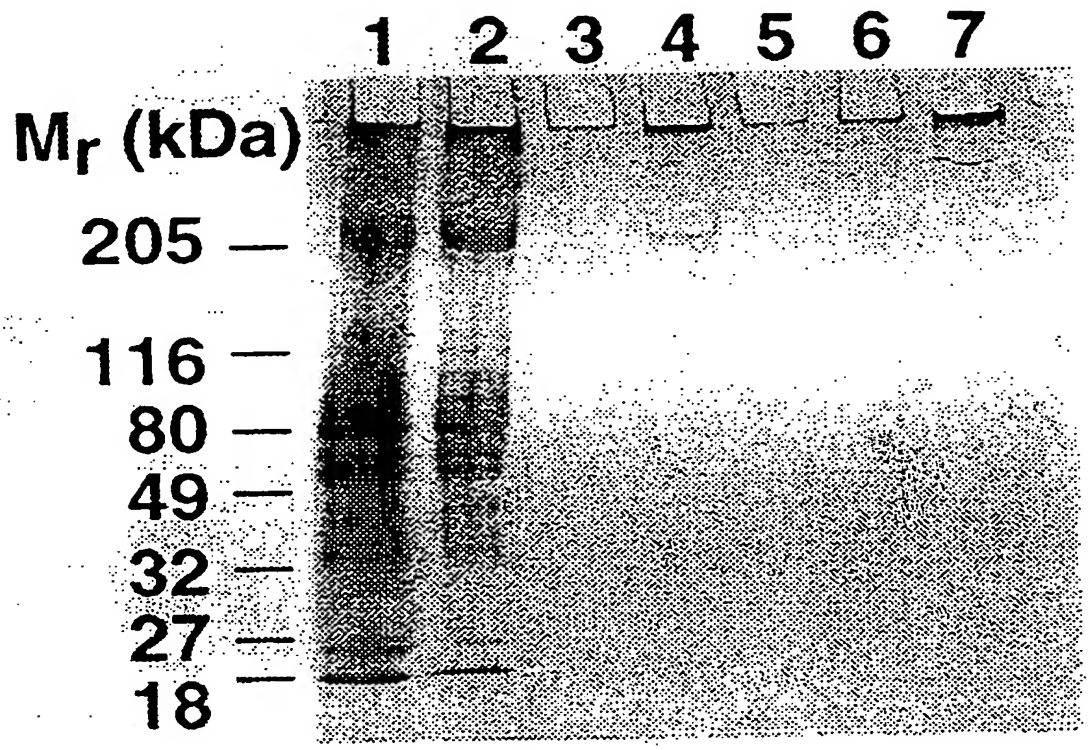


FIGURE 2.

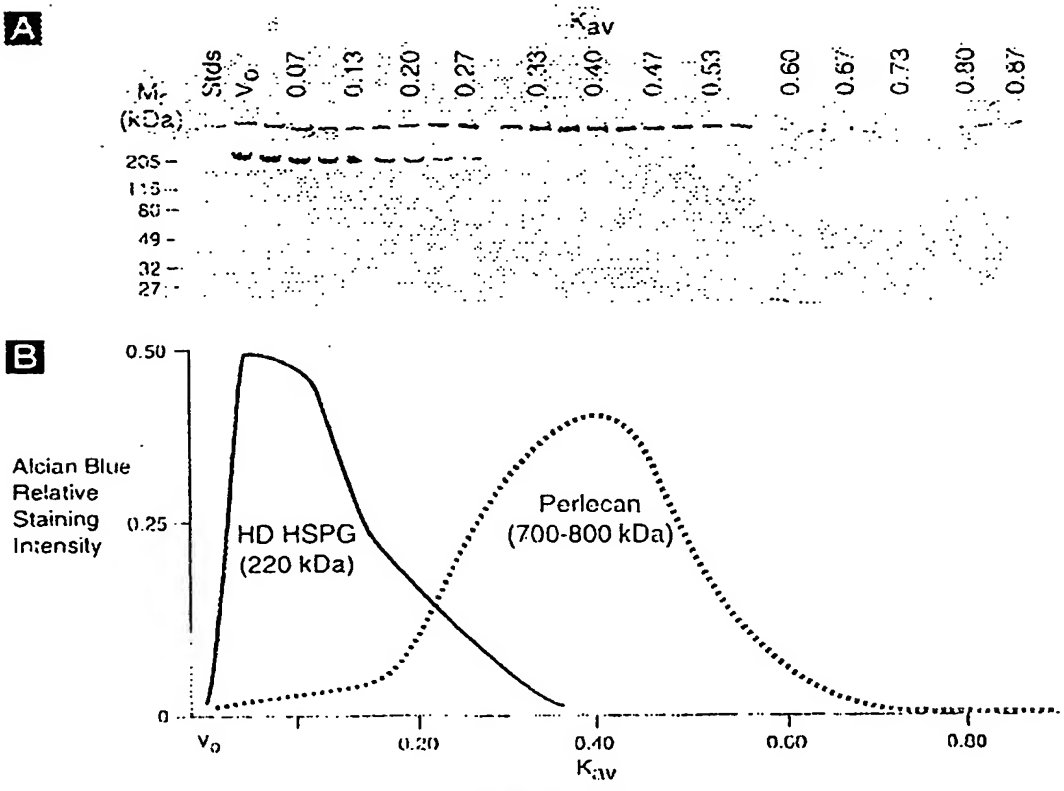


FIGURE 3

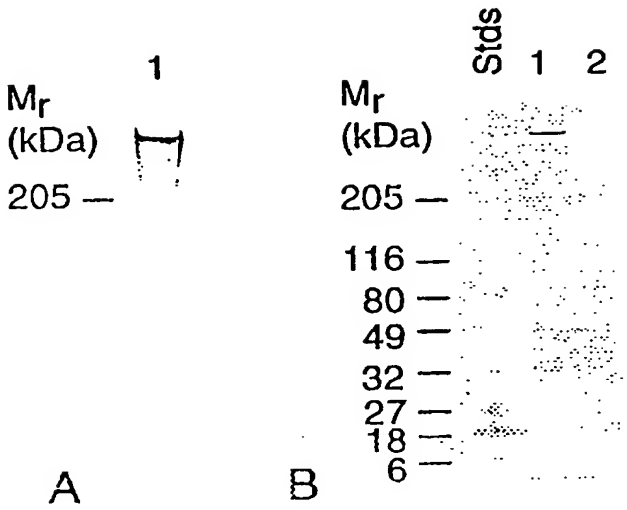


FIGURE 4

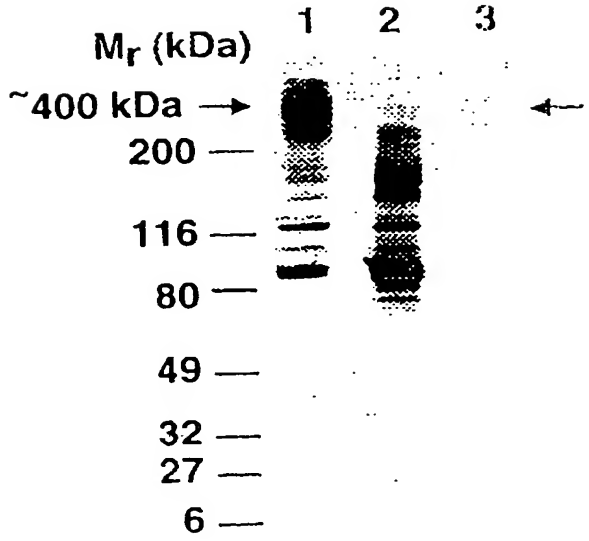


FIGURE 5

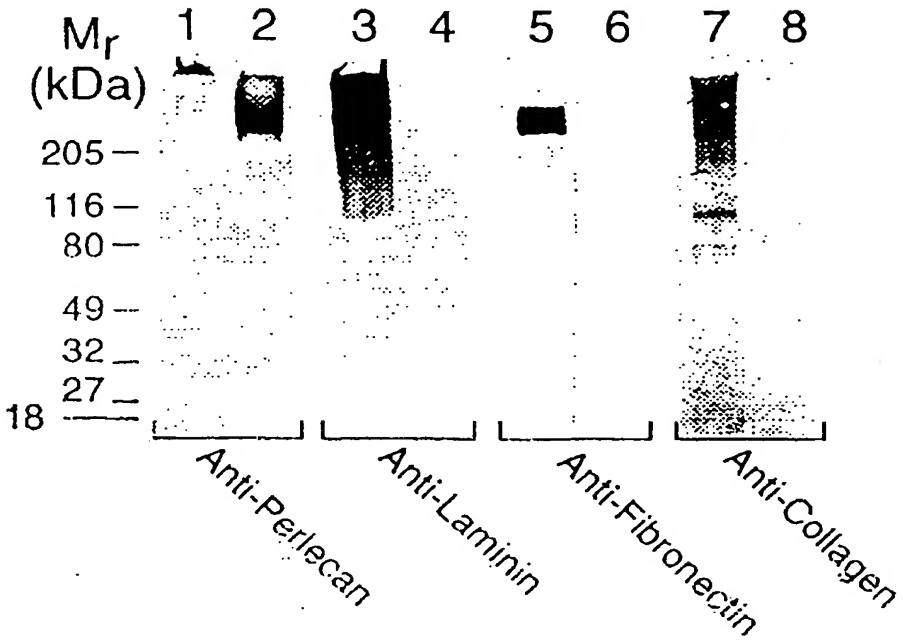


FIGURE 6

FIGURE 8a

FIGURE 8b

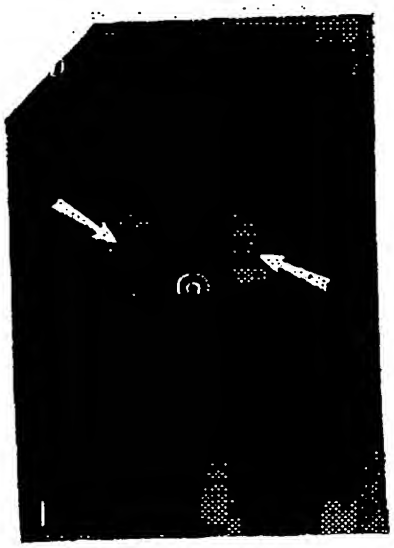


FIGURE 8c

Pre-extract 50 Grams EHS Tumor with 50 mM Tris-HCl, 3.4 M NaCl and protease inhibitors then Centrifuge at 17,000 x g for 30 min

↓

Multiple Extractions of Pellet in 4 M Guanidine-HCl containing 0.5 % CHAPS and Protease Inhibitors then Centrifuge at 17,000 x g for 30 min

↓

Collect Supernatants

↓

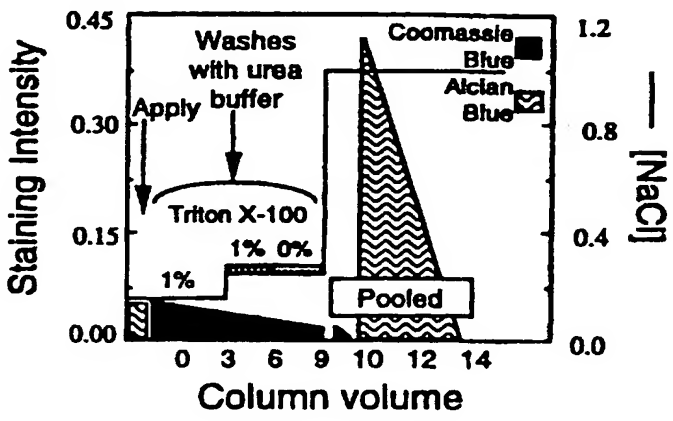
Apply to Sephacryl S-400 Column and Elute with 7 M Urea, 0.2 M NaCl, 0.1 % CHAPS, 50 mM Tris (pH 8.0) containing Protease Inhibitors

↓

Apply Void Volume Fractions to a 50 ml SP-Sepharose

↓

Apply Non-binding Fractions to a 50 ml DEAE-Sephacel Column



↓

Apply PG Fractions to Sephacryl S-1000 Column and Elute with 1 M Urea, 50 mM Tris-HCl (pH 8.0) containing Protease Inhibitors. Redirect the Eluate having Kav = 0.29 - 0.54 to an 8 ml Heparin-Sepharose Column Before Fraction Collection

↓

Repeat the S-1000 column fractionation on the unpure fractions if necessary

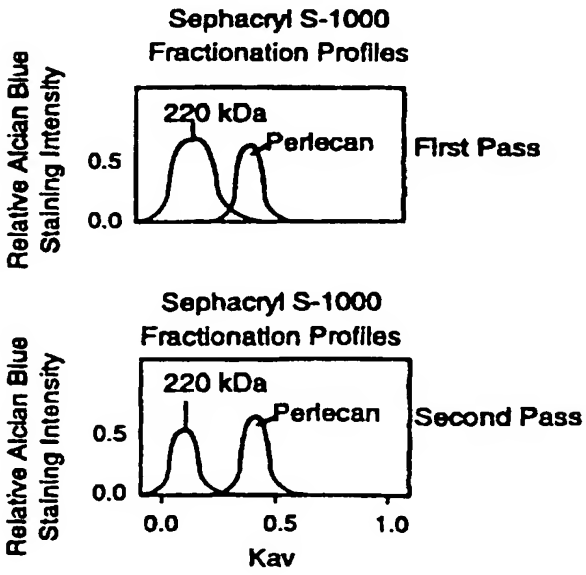


FIGURE 7

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/04422

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/9.1; 435/7.1; 436/547, 548; 514/2; 530/350, 412, 413, 415, 416, 417; 800/2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ----- Y	CASTILLO et al. Purification and detailed characterization of perlecan isolated from the engelbreth-holm-swarm (EHS) tumor for use in an animal model of Abeta amyloid persistence in brain. Society for Neuroscience. 11 November 1995, Vol. 21, Part 3, page 1724, see entire abstract 673.15.	1-14 -------- 15, 16, 21, 22, 26-30
Y	SNOW et al. An important role of heparan suflate proteoglycan (perlecan) in a model system for the deposition and persistence of fibrillar Abeta-amyloid in rat brain. Neuron, January 1994, Vol. 12, pages 219-234.	26-30

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/04422

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	HOLCOMB et al. Toxicity of Abeta peptide variants in rat brain. Society for Neuroscience. 11 November 1995, Vol. 21, page 474, see entire abstract 193.4.	26-30
X	AVIEZER et al. Perlecan, basal lamina proteoglycan, promotes basic fibroblast growth factor-receptor binding, mitogenesis, and angiogenesis. Cell. 16 December 1994, Vol. 79, pages 1005-1013, see espically pages 1008-1010.	24, 25
Y	MURDOCH et al. Widespread expression of perlecan proteoglycan in basement membranes and extracellular matrices of human tissues as dietected fy a novel monoclonal antibody against domain III in by in situ hybridization. The Journal of Histochemistry and Cytochemistry. 1994, Bol. 42, No. 2, pages 239-249, see entire document.	15-22
Y	MARESH et al. Detection and quantitation of perlecan mRNA levels in Alzheimer's disease and normal aged hippocampus by competitive reverse transcription-polymerase chain reaction. Journal of Neurochemistry. September 1996, Vol. 67, No. 3, pages 1132-1144, see entire document.	17-21

INTERNATIONAL SEARCH REPORT

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A. CLASSIFICATION OF SUBJECT MATTER:
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A. CLASSIFICATION OF SUBJECT MATTER:
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search terms: animal models, perlecan, antibody, immunoglobulin, immunization, administration, diagnosis, levels, Alzheimer's Disease, purification, isolation, chromatography.

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(54) Title: SEQUESTRATION OF Aβ IN THE PERIPHERY IN THE ABSENCE OF IMMUNOMODULATING AGENT AS A THERAPEUTIC APPROACH FOR THE TREATMENT OR PREVENTION OF BETA-AMYLOID RELATED DISEASES

(57) Abstract: The present invention describes a method of administering an Aß-binding agent or drug which has affinity for amyloid beta (Aß) in the periphery (blood) and reducing Aß levels in the brain without the need for the agent or drug to enter the brain itself. The Aß-binding agents utilized in the methods of the invention are preferably non-immunomodulating agents (e.g., antigenic peptides or antibodies) and bind to Aß in the periphery, or blood. Such compounds do not significantly cross the blood/brain barrier, and yet they lower amyloid (Aß) levels in the brain, thereby serving as safer, therapeutic and prophylactic treatments against diseases associated with Aß in the brain, e.g., Alzheimer's Disease and amyloid angiopathy, as well as against other AD-related amyloidoses.

SEQUESTRATION OF Aβ IN THE PERIPHERY IN THE ABSENCE OF IMMUNOMODULATING AGENTS AS A THERAPEUTIC APPROACH FOR THE TREATMENT OR PREVENTION OF BETA-AMYLOID RELATED DISEASES

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FIELD OF THE INVENTION

The present invention relates to improved drug delivery methods and the discovery and development of novel compounds and drugs for the treatment and prevention of neurological diseases and disorders associated with β-amyloid, such as Alzheimer's disease, β-amyloid related problems in Down's syndrome and vascular dementia (cerebral amyloid angiopathy) and other amyloidosis diseases. The invention further relates to diagnostic and screening methods for determining or identifying the aforementioned diseases and disorders associated with β-amyloid in patients.

BACKGROUND OF THE INVENTION

Alzheimer's Disease (AD) is the most common cause of chronic dementia, with approximately two million people in the United States having the disease. The histopathologic lesions of Alzheimer's disease (i.e., neuritic amyloid plaques, neurofibrillary degeneration, and granulovascular neuronal degeneration) are found in the brains of elderly people with Alzheimer's dementia.

It is estimated that ten percent of individuals older than 65 years of age have mild to severe dementia. The number of such lesions correlates with the degree of intellectual deterioration. This high prevalence, combined with the rate of growth of the elderly segment of the population,

make dementia (and particularly AD) one of the most important of the present-day public health concerns.

An invariant feature of Alzheimer's disease (and AD in Down's syndrome) is the deposition of the small, i.e., approximately 40 to 42 residues, amyloid beta (also referred to as Aβ or Abeta herein) peptide as insoluble β-amyloid plaque in the brain parenchyma. (G.G. Glenner et al., 1984, *Appl. Pathol.*, 2(6):357-69; G.G. Glenner et al., 1984, *Biochem Biophys Res Commun.*, 120(3):885-90; G.G. Glenner et al., 1984, *Biochem Biophys Res Commun.*, 122(3):1131-5). In cerebral amyloid angiopathy, Aβ is deposited in the vasculature. Aβ is generated by proteolysis of the approximately 100 kDa amyloid precursor protein (APP), a broadly expressed type-1 transmembrane protein that is found primarily in the trans-Golgi network (TGN) and at the cell surface (reviewed in B. De Strooper and W. Annaert, 2000, "Proteolytic processing and cell biological functions of the amyloid precursor protein." *J. Cell. Sci.*, 113(Pt 11)(7):1857-1870). The β-amyloid precursor protein APP is further described in D.J. Selkoe et al., 1988, *Proc. Natl. Acad. Sci. USA.*, 85(19):7341-7345; R.E. Tanzi et al., 1988, *Nature*, 331(6156):528-530; and E. Levy et al., 1990, *Science*, 248(4959):1124-1126.

Amyloid plaques containing Abeta (Aβ) peptides are one of the most significant pathological features of the human Alzheimer's disease brain. Drugs that reduce brain Aβ levels, or remove plaques, are considered to be the most likely to be effective in the treatment or prevention of AD. To date, treatments for AD have focused on the use of anti-Aβ antibodies or peptides which evoke the production of anti-Aβ antibodies, i.e., vaccine therapy. (see, for example, D. Schenk et al., 1999, *Nature*, 400(6740):173-177 and F. Bard et al., 2000, *Nat Med*, 6(8):916-919).

The presumed mode of action for such antibody, or immunomodulatory, treatments is in the clearance of Aβ directly from the brain due to the entry of antibodies into the brain. A side effect of vaccination is an increase of peripheral Aβ levels as levels of Aβ decrease in the brain, thus resulting in a little understood change in the dynamics between the two systems, brain and periphery.

Vaccination involving anti-Aβ antibodies is a potentially ineffective and possibly even dangerous approach for treatment of AD patients, particularly the elderly who lack (or have less) immune responsiveness, due to the risk of provoking autoimmune diseases.

Clearance of Aβ from the brain has been reported using immunization of Aβ peptides or passive immunization with anti-Aβ antibodies (D. Schenk et al., 1999, *Nature*, 400(6740):173-177 and F. Bard et al., 2000, *Nature Med.*, 6(8):916-919). The proposed mechanism for this clearance is microglial phagocytosis carried out by brain immune cells, i.e., microglia, that have been activated by elicited or injected anti-Aβ antibodies.

A goal in the field of therapy and prevention of AD and amyloid-related diseases is the discovery and development of new drugs that are effective due to their mode of action in the periphery, rather than in the brain, thus obviating the need to enter the brain itself and overcoming the problems encountered in efficient dosage and effectiveness due to the blood/brain barrier. The present invention satisfies this goal by providing new methods of treatment and prevention for AD and other amyloid-related diseases, and by describing new types of drugs and compounds that serve to treat or prevent disease via the blood.

SUMMARY OF THE INVENTION

The present invention provides methods and compounds (also termed drugs, substances, reagents, or agents, preferably bioactive agents) employed therein for sequestering Aβ in the blood, or blood components, such as plasma, i.e., the periphery, thereby reducing Aβ levels in the brain for treatment or prevention of beta-amyloid related diseases. The compounds of the invention have an affinity for (i.e., "sequester") Aβ, and bind to and sequester Aβ in the blood, or periphery, e.g., plasma, without needing to enter the brain itself. According to this invention, such compounds do not (and do not need to) cross the blood/brain barrier, and yet they significantly lower amyloid (Aβ) levels in the brain. Such compounds have been shown in animal models of disease, e.g., a transgenic AD mouse model, to lower Aβ levels in the brain by sequestering Aβ in the periphery, e.g., plasma. That the invention provides a method and drugs used therein which obviate the need for a drug to enter the brain itself, while still significantly lowering amyloid (Aβ) levels in the brain, offers a great improvement over drugs that currently must enter the brain to have an effect on Aβ levels in the brain.

Thus, it is also an aspect of the present invention to provide a method of treating or preventing AD comprising administering to an individual in need thereof a compound or drug having an affinity for Aβ, which binds to Aβ in the periphery, wherein such a compound or drug, preferably a non-immune related compound or drug, and also preferably, an agent other than an antibody or an immunomodulating agent, sequesters Aβ in the periphery and leads to a reduction in Aβ levels in the brain. For delivery to the periphery, such Aβ-binding compounds are preferably introduced intravenously or subcutaneously; however, any method of

introducing the compound into the blood stream (including via pumps) is acceptable and suitable in accordance with this invention.

It is another aspect of the present invention to provide a method of reducing amyloid (or soluble/insoluble Aβ) levels in the brain of a patient undergoing treatment by obviating the need to introduce an Aβ-binding drug or compound directly, or indirectly, into the brain. According to the invention, the effectiveness of the method in which the Aβ-binding drug sequesters Aβ in the bloodstream and removes it from the brain is at least as high as a vaccine approach involving the production of antibodies that cross the blood/brain barrier, enter into the brain, and act in the brain.

It is yet a further aspect of the present invention to provide a method for diagnosing, screening, or monitoring treatment of diseases involving β-amyloid or amyloidoses, such as AD, comprising determining an elevation of Aβ levels in the periphery, e.g., plasma, particularly at a time, or series of times, following treatment. In accordance with this aspect of the invention, the elevation of Aβ levels in the periphery, e.g., plasma, serves as a diagnostic marker of diseases involving β-amyloid, particularly, AD.

Further aspects, features and advantages of the present invention will be apparent when considered in connection with the further disclosure of the invention hereinbelow.

DESCRIPTION OF THE INVENTION

In one of its aspects, the present invention describes compounds (drugs) which have an affinity for, i.e., "sequester", Aβ in the blood, or blood components, e.g., plasma, (periphery) and which reduce Aβ levels in the brain without the need of the compounds (e.g., drugs or bioactive agents) to enter the brain itself. Such compounds sequester Aβ in

the periphery and alter the periphery/brain dynamics so as to reduce Aβ in the brain by virtue of their effective sequestration of Aβ in the periphery.

According to this invention, such compounds are preferably brain impermeable and essentially do not (and do not need to) cross the blood/brain barrier following administration or introduction into a recipient, and yet they significantly lower amyloid (Aβ) levels in the brain. Also in accordance with this invention, such compounds have been shown in animal models of disease, e.g., AD mouse models, to lower Aβ levels in the brain by sequestering Aβ in the periphery, e.g., plasma. (Example 1). It is thus an aspect of the invention that the Aβ binding agent, drug, compound, and the like, effectively sequesters Aβ in the periphery following administration in the periphery. Preferably, greater than about 50% of the Aβ binding agent, drug or compound remains in the periphery versus the brain following administration in the periphery. More preferably, about 90% or more of the Aβ binding agent, drug or compound remains in the periphery versus the brain following administration in the periphery

In addition, the finding of elevated Aβ in the periphery, particularly in plasma, preferably in conjunction with the administration of agents that bind Aβ and sequester Aβ in the periphery, can serve as a diagnostic marker of β-amyloid-related diseases, especially, AD. The elevation of levels of Aβ in the periphery can further serve as a means of monitoring the effectiveness of treatment of a disease involving Aβ, particularly with a drug or agent that binds and sequesters Aβ in the periphery, thereby leading to its elevation in the periphery. According to this embodiment of the invention, an elevation of Aβ in the periphery reflects an amount of Aβ that is increased relative to that found in normal individuals, such as in plasma, or a base level of Aβ, e.g., in plasma, in individuals who

serve as controls. Determining and/or measuring levels of Aβ can be performed using routine techniques as known in the art, such as radioimmunoassays (RIAs), non-radioactive immunoassays, such as enzyme linked immunoassays (ELISAs), western blotting, dot blotting, mass spectrometry, etc.

Without wishing to be bound by theory, it is proposed that microglial phagocytosis is not necessary for Aβ clearance from the brain in accordance with the present invention. Instead, sequestration in the blood, or blood component, such as plasma, i.e., the periphery, in the absence of an immune modulating agent, by suitable Aβ-binding compounds that are not Aβ peptides or their derivative antibodies, serves to reduce Aβ levels in the brain and to alter the central nervous system (CNS)/periphery dynamics leading to reduction of Aβ in the brain. As used herein, the terms immune modulating agent, and immune related agent, refer to an anti-Aβ antibody or a peptide against some region of Abeta or APP that evokes the production of antibodies, which recognize an Abeta region or APP.

As a consequence of the sequestration of Aβ in the periphery according to this invention, higher levels of Aβ are predicted to be found in the blood (e.g., plasma)/periphery), (see Example 1), because Aβ is sequestered in the blood/periphery by the Aβ-binding compounds. It is also to be understood that the removal of bound Aβ/binding agents by cellular clearance mechanisms may effectively reduce the levels of peripheral Aβ seen following administration of the sequestering agent. The important effect of the methods and reagents of the present invention is that the levels of Aβ found in the brain as a result of keeping Aβ sequestered in the periphery are reduced, which is advantageous for the therapeutic effect of the method and compounds of the present invention.

The invention also allows for determining or monitoring a drug's effectiveness by monitoring Aβ levels in the periphery, such as in the plasma, instead of, or in addition to, brain Aβ levels. (see, e.g., Example 1). Methods of monitoring Aβ levels in an individual undergoing drug treatment or therapy for amyloid related diseases involve determining the levels of Aβ in the individual's peripheral body fluid sample, e.g., plasma, at one or more time intervals following treatment or therapy involving an Aβ binding agent that sequesters Aβ in the periphery. For example, an individual can be monitored at about 1-25 hours, preferably at about 2-10 hours following administration of the Aβ binding agent, or at varying time intervals therebetween, to determine if Aβ levels are elevated. Such monitoring methods are particularly useful for determining if a given drug treatment is beneficial, or to determine if doses of a drug or a drug combination should be modified or adjusted during the course of treatment. In such methods, it is preferred to use the individual's pretreatment levels of Aβ in the periphery to compare and assess treatment and post-treatment levels of Aβ in the periphery. Controls can also include peripheral levels of Aβ in disease-free (or dementia-free) individuals, as well as peripheral levels of Aβ in individuals having an amyloid related disease, e.g., AD. Human plasma and cerebrospinal fluid levels of amyloid beta proteins, particularly, Aβ40 and Aβ42, have been reported (see, e.g., Mehta et al., 2000, *Arch. Neurol.*, 57:100-105).

According to this invention, Aβ levels can be measured in an individual's body fluid sample, such as blood, serum, or plasma, using conventionally known assays that detect Aβ, for example, radioisotopic immunoassays or non-isotopic immunoassays, e.g., fluorescent immunoassays, chemiluminescent immunoassays and enzymatic immunoassays, such as an enzyme linked immunoassay (ELISA), as are

commercially available, known and practiced in the art, for example, Beta-amyloid (Abeta) [1-40] Immunoassay (Biosource, Camarillo, CA; Cat. No. KHB3481); Beta-amyloid (Abeta) [1-42] Immunoassay (Biosource, Camarillo, CA; Cat. No. KHB3441); and Human Amyloid beta (1-40) Immunoassay (IBL, Fujioka, Gunma, Japan; Cat. No. 17713).

Typically, an ELISA assay initially involves preparing, obtaining, or employing an antibody specific to Aβ, preferably a monoclonal antibody. In addition, a reporter antibody is used. In some ELISA protocols, the reporter antibody recognizes and binds to the anti-Aβ-specific monoclonal antibody. To the reporter antibody is attached a detectable reagent such as a radioactive isotope, a fluorescent moiety, a chemiluminescent moiety, or, in an ELISA, an enzyme, such as horseradish peroxidase or alkaline phosphatase.

As is appreciated by those skilled in the art, ELISAs can be performed in a number of assay formats. In one ELISA format, a host sample, e.g., a patient body fluid sample, is incubated on a solid support, e.g., the wells of a microtiter plate, or a polystyrene dish, to which the proteins in the sample can bind. Any free protein binding sites on the dish are then blocked by incubating with a non-specific protein such as bovine serum albumin. The monoclonal antibody is then added to the solid support, e.g., the wells or the dish, and allowed to incubate. During the incubation time, the monoclonal antibodies attach to any Aβ polypeptides or peptides that have attached to the polystyrene dish.

All unbound monoclonal antibody is washed away using an appropriate buffer solution. The reporter antibody, e.g., linked to horseradish peroxidase, is added to the support, thereby resulting in the binding of the reporter antibody to any monoclonal antibody which has bound to Aβ present in the sample. Unattached reporter antibody is then

washed away. Peroxidase substrate is added to the support and the amount of color developed in a given time period provides a measurement of the amount of Aβ that is present in a given volume of individual or patient sample when compared to a standard curve.

In another ELISA format, antibody specific for a particular analyte is attached to the solid support, i.e., the wells of a microtiter plate or a polystyrene dish, and a sample containing analyte is added to the substrate. Detectable reporter antibodies, which bind to the analyte that has bound to the capture antibodies on the support, are then added, after the appropriate incubations and washings, and analyte-antibody complexes are detected and quantified.

The present invention also embraces a sandwich type ELISA immunoassay typically performed using microtiter plates. A capture antibody, that can be polyclonal or monoclonal, preferably a monoclonal antibody, that specifically recognizes an epitope in the Aβ peptide is used, along with a labeled detector antibody, e.g., an alkaline phosphatase-labeled antibody, or a horse radish peroxidase-labeled antibody, preferably a monoclonal antibody. The detector antibody also specifically recognizes an epitope in Aβ. Preferably also, the capture antibody does not inhibit binding to Aβ. The production of both polyclonal and monoclonal antibodies, particularly monoclonal antibodies that are specific for Aβ, is performed using techniques and protocols that are conventionally known and practiced in the art.

In a particular embodiment according to this invention, a capture anti-Aβ antibody of the assay method is immobilized on the interior surface of the wells of the microtiter plate. To perform the assay, an appropriate volume of sample is incubated in the wells to allow binding of the antigen by the capture antibody. The immobilized antigen is then

exposed to the labeled detector antibody. Addition of substrate to the wells, if the detectable label is alkaline phosphatase, for example, allows the catalysis of a chromogen, i.e., *para*-nitrophenylphosphate (pNPP), if the label is alkaline phosphatase, into a colored product. The intensity of the colored product is proportional to the amount of Aβ that is bound to the microtiter plate.

Standards are used to allow accurate quantitative determinations of Aβ in the samples undergoing analysis. A microtiter plate reader simultaneously measures the absorbance of the colored product in the standard and the sample wells. Correlating the absorbance values of samples with the standards run in parallel in the assay allows the determination of the levels of Aβ in the sample. Samples are assigned a quantitative value of Aβ in nanograms per milliliter (ng/ml) of blood, serum, plasma, or other body fluid.

The present invention provides a significant advantage to the treatment and prevention of AD and amyloid-related diseases in that drugs and active compounds according to this invention are not required to cross the blood/brain barrier to exert their effect. Having to cross the blood/brain barrier is an enormous obstacle to developing effective drugs for use in the brain. This invention overcomes this obstacle. One of the major differences between this method and others is that it uses a non-antibody compound, or a compound that is not related to an antibody, to achieve the sequestration of Aβ, and that this sequestration has its primary effect in the periphery. A consequence of the method and the compounds utilized therein is a decrease in Aβ in the brain.

In addition, a second advantage of this invention is that neither Aβ peptides, nor anti-Aβ antibodies, is administered to a host, thus negating the risk of an adverse immune response, or the lack of an effective immune

response. For AD and amyloid angiopathy, this method preferably involves the use of Aβ-binding compounds and drugs of the invention, more preferably formulated as pharmaceutically acceptable compositions as described herein.

The method further comprises the administration of an amyloid beta (Aβ)-binding compound, agent, or drug in the periphery of an individual in need thereof, wherein the compound sequesters Aβ in the periphery and concomitantly decreases Aβ levels in the brain of the individual undergoing treatment. According to the method of the present invention, the need to introduce an agent or drug directly into the brain, or to have the drug cross the blood/brain barrier is obviated. Also according to this method, brain Aβ levels are reduced, due to the effects of the Aβ-binding drugs and compounds described herein on Aβ in the periphery.

The method and Aβ-binding compounds and agents used therein in accordance with the present invention are suitable for the treatment, both prophylactic and therapeutic, of neurological diseases and disorders associated with β-amyloid, such as Alzheimer's disease, β-amyloid related problems in Down's syndrome and vascular dementia (cerebral amyloid angiopathy) (A.J. Rozemuller et al., 1993, *Am. J. Pathol.*, 142(5):1449-1457) and other amyloidosis diseases. The method involving peripheral sequestration of disease associated agents, e.g., peptides, or proteins or aggregates thereof, by non-immunomodulating agents that bind to such disease associated agents in the blood/periphery in accordance with the present invention, are also useful in the treatment or prevention of other cortical or vascular amyloidoses, including those caused by cystatin C (ACys), prion protein (AScr), transthyretin (ATTR), gelsolin (AGel), and Amyloid ABri (or A-WD) (see, M. Yamada, 2000, "Cerebral amyloid

angiopathy: an overview", *Neuropathology*, 20(1):8-22). Cortical or vascular amyloidoses are very similar in etiology to AD.

In accordance with the present invention, the method of delivering Aβ-binding drugs to the periphery has been shown to be at least as effective as the vaccine approach in transgenic mouse models, if not more so. Indeed, the use of non-toxic, non-immune related compounds and drugs can overcome adverse immune responses that are frequently associated with the use of brain-directed immunovaccines. Prior to the present invention, the treatment of brain amyloidosis by administering non-immune related Aβ-binding agents in the periphery and sequestering or "locking away" Aβ in the blood, or periphery, has not been shown.

Suitable compounds that can be employed in the method of this invention include, but are not limited to, small molecules, e.g., peptides, proteins; biologic agents; and drugs that have an affinity for Aβ and bind Aβ in the periphery. Such compounds, molecules, agents and drugs have an Aβ-binding domain that physically binds to and locks away Aβ in the periphery. The compound, molecule, agent or drug can bind to or have affinity for a variety of Aβ peptides, e.g., Aβ peptides derived from APP; Aβ peptides of different fragment lengths, e.g., Aβ40 or Aβ42, and the like. In addition, the compound, molecule, agent or drug can bind to or have affinity for any portion of an Aβ peptide, e.g., the N- or C- terminus, or other regions of the molecule. Non-immune related and/or non-immunomodulatory compounds or drugs are preferred. Most preferably the compounds are non-toxic and well tolerated following their use in the treatment and prevention methods.

An advantage of the use of molecules other than immune related compounds, such as antibodies, for peripheral sequestration of

Abeta (Aβ) is that non-antibody related drugs can be manipulated more easily than antibodies. For example, sequestering compounds can be modified to be metabolized faster by the addition of certain chemical structures, as known and practiced in the art. For example, sequestering compounds can be modified by the addition of side chain(s) which can modulate metabolism. Such chemical modification of the non-antibody Aβ-binding and sequestering compounds can improve their efficacy and reduce toxicity and/or potentially adverse side effects. The derivative of Congo Red, an Aβ-imaging agent, as described herein, is particularly suitable for chemical derivatization or modification.

Nonlimiting examples of such Aβ-binding compounds include compounds having an affinity for Aβ, particularly, cortigangliosides, such as GM1, the actin-regulating molecule gelsolin, particularly, the extracellular Aβ-binding domain of gelsolin, and Aβ staining molecules, such as derivatives of Congo Red, e.g., [1,4-bis(3-carboxy-4-hydroxyphenylethenyl)-benzene and 5,5'-[(1,1' biphenyl)-4, 4'-diylbis(azo)] bis [2-hydroxybenzoic acid] disodium salt (chrysamine-G or CG), as described in U.S. Patent No. 6,133,259 and WO 96/34853. A preferred Aβ staining molecule is the Aβ staining dye compound Chrysamine-G, as described in U.S. Patent No. 6,133,259 and WO 96/34853. (Example 2). Other nonlimiting examples of Aβ binding agents that are suitable for use in the methods of this invention include Aβ imaging agents (e.g., Klunk et al., 1995, "Chrysamine-G binding to Alzheimer and control brain: autopsy study of a new amyloid probe", *Neurobiol. Aging*, 16: 541-548), β-sheet breakers (e.g., Bohrmann et al., 2000, "Self-assembly of beta-amyloid 42 is retarded by small molecular ligands at the stage of structural intermediates", *J. Struct. Biol.*, 130:232-246), β-sheet formation inhibitors (e.g., Findeis et al., 1999, "Modified-peptide inhibitors of amyloid beta-peptide polymerization", *Biochemistry*,

38:6791-6800), and the like, are encompassed for use in the present invention.

The Aβ-binding compounds according to the invention can be incorporated into pharmaceutical formulations, or pharmaceutical compositions, preferably physiologically acceptable compositions, according to known methods, such as by admixture with a pharmaceutically acceptable carrier, diluent, or excipient. One or more Aβ-binding compounds or drugs comprise the pharmaceutical compositions and are formulated as active ingredients in the compositions in a therapeutic or prophylactic amount.

The pharmaceutically, or physiologically, acceptable carrier, diluent, or excipient can be any compatible non-toxic substance suitable to deliver the compound to a host or recipient. Sterile water, alcohol, fats, waxes and inert solids may be used as carriers. In addition, pharmaceutically acceptable adjuvants, buffering agents, dispersing agents, and the like, may also be incorporated into the pharmaceutical compositions. The preparation of pharmaceutical compositions comprising active agents is well described in the scientific and medical literature. Examples of methods of formulation, and carriers, etc. may be found in the latest edition of *Remington's Pharmaceutical Sciences,* 18th Ed., 1990, Mack Publishing Co, Easton, PA.

To formulate a pharmaceutically acceptable composition suitable for effective administration, preferably *in vivo*, or even *ex vivo*, such compositions will contain an effective amount of the active compound, biomolecule, agent or drug. Pharmaceutical compositions of the present invention are administered to an individual in amounts effective to treat or prevent AD, amyloid angiopathy, or other Aβ-associated diseases or conditions. The effective amount may vary according to a variety of factors,

such as an individual's physical condition, weight, sex and age. Other factors include the mode and route of administration. These factors are realized and understood by the skilled practitioner and are routinely taken into account when administering a therapeutic agent to an individual.

Pharmaceutical compositions suitable for use in the present invention include compositions wherein the active ingredients are contained in an effective and sufficient amount to directly bind Aβ in the periphery, sequester it there, and reduce the Aβ levels in the brain. The determination of an effective dose is well within the capability of the skilled practitioner in the art. The therapeutically effective dose can be estimated initially either in cell culture assays, or in animal models, usually mice, rabbits, dogs, pigs, rats, monkeys, or guinea pigs. The animal model is also used to achieve a desirable concentration range and route of administration. Such information can then be used to determine useful doses and routes for administration in humans.

A therapeutically effective dose refers to that amount of the Aβ-binding compound or drug which ameliorates, reduces, or eliminates the symptoms or condition. In accordance with this invention, the effective dose is preferably that which lowers, reduces, or eliminates levels of Aβ, or buildup of Aβ, in the brain, while binding to and "locking up" Aβ in the periphery. The exact dosage is chosen in view of the patient to be treated, the route of administration, the severity of disease, and the like.

The concentration of the Aβ-binding drug, compound or bioactive agent in the pharmaceutical carrier may vary, e.g., from less than about 0.1% by weight of the pharmaceutical composition to about 20% by weight, or greater. As a nonlimiting example, a typical pharmaceutical composition for intramuscular administration would be formulated to contain one to four milliliters (ml) of sterile buffered water and one microgram (μg) to

one milligram (mg) of the Aβ-binding drug or compound of the present invention. A typical composition for intravenous infusion could be formulated to contain, for example, 100 to 500 ml of sterile buffered water or Ringer's solution and about 1 to 100 mg of the Aβ-binding drug or compound.

The daily dosage of the pharmaceutical, or physiologically acceptable, products may be varied over a wide range, for example, from about 0.01 to 1,000 mg per adult human/per day. An effective amount of the drug is ordinarily supplied at a dosage level of from about 0.0001 mg/kg to about 100 mg/kg of body weight per day. The range is more particularly from about 0.001 mg/kg to 10 mg/kg of body weight per day. Even more particularly, the range varies from about 0.05 to about 1 mg/kg. Of course, it will be understood by the skilled practitioner that the dosage level will vary depending upon the potency or effectiveness of a particular compound, or combination of compounds, and that certain compounds will be more potent or effective than others.

In addition, the dosage level will vary depending upon the bioavailability of the compound. The more bioavailable and potent the compound, the less amount of the compound will need to be administered through any delivery route, including, but not limited to, oral delivery. The dosages of the Aβ-binding compounds are adjusted, if combined, in order to achieve desired effects. On the other hand, dosages of the various Aβ-binding agents or compounds may be independently optimized and combined to achieve a synergistic result, wherein the pathology is reduced more than it would be if one single agent or compound were used alone.

The pharmaceutical compositions may be provided to an individual in need of therapeutic treatment by a variety of routes, such as; for example, subcutaneous, topical, oral, intraperitoneal, intradermal,

intravenous, intranasal, rectal, intramuscular, and within the pleural cavity. Administration of pharmaceutical compositions is accomplished orally or parenterally. More specifically, methods of parenteral delivery include topical, intra-arterial, intramuscular, subcutaneous, intramedullary, intrathecal, intraventricular, intravenous, intraperitoneal, intranasal administration, or via the pleural cavity. In addition, the compounds according to the invention can be delivered via one or more routes of administration through the use of pumps.

Also embraced are transdermal modes of delivery, such as patches and the like, with or without a suitable permeation enhancer. The methods and compositions embodied by the invention provide a means by which one or more of the Aβ-binding drugs, or medicaments, can be effectively administered in a transdermal system. Frequently, compounds having poor topical absorption, or which are required at high dosage levels, are delivered transdermally. Accordingly, a transdermal means of delivering a drug composition (often with a permeation enhancing composition) to the skin is that of the transdermal patch or a similar device as known and described in the art. Examples of such devices are disclosed in U.S. Patent Nos. 5,146,846, 5,223,262, 4,820,724, 4,379,454 and 4,956,171. The transdermal mode of storing and delivering the compositions onto the skin and forming the active composition is convenient and well suited for the purposes of the invention.

The present invention also provides suitable topical, oral, systemic and parenteral pharmaceutical formulations for use in the methods of treatment described herein. It is to be appreciated that the compositions containing the Aβ-binding compounds can be administered in a wide variety of therapeutic dosage forms in conventional vehicles for administration. For example, the compounds can be administered in such oral dosage forms as

tablets or capsules (including timed release and sustained release formulations), pills, powders, granules, elixirs, tinctures, solutions, suspensions, syrups and emulsions, or by injection. Likewise, the therapeutic compounds may also be administered in intravenous (both bolus and infusion), intraperitoneal, subcutaneous, topical (with or without occlusion), or intramuscular form, all using forms well known to those of ordinary skill in the pharmaceutical arts. The preferred mode of delivery for the Aβ-binding compounds according to the present invention is intravenous.

For topical administration, the compositions of the present invention may be formulated in oil, water, or combinations thereof. Preferred is a dermatologically acceptable formulation comprising an oil-in-water emulsion. Examples of other dermatologically acceptable vehicle formulations of the present invention include, but are not limited to, any suitable non-toxic or pharmaceutically acceptable topical carrier, such as a solution, suspension, emulsion, lotion, ointment, cream, gel, plaster, patch, film, tape or dressing preparation, all of which are well-known to those skilled in the art of topical skin formulations and preparations.

The pharmaceutical compositions of the present invention can be administered for therapeutic and/or for prophylactic purposes of treating diseases, pathologies, or conditions related to the increase in Aβ levels, or the deposition of Aβ in the brain, for example, AD and amyloid angiopathy. Prophylactic treatment is preferred, although therapeutic treatment is also efficacious. For prophylactic applications, the pharmaceutical compositions of this invention are administered to an individual who is susceptible to, or prone to, the disease, pathology, or condition. Such individuals can be identified by genetic screening and/or clinical analysis, such as is described in the medical literature (see, e.g., Goate, 1991, *Nature*, 349:704-706 and

E.H. Corder et al., 1993, *Science*, 261(5123):921-923). In such cases, the pharmaceutical compositions bind to or sequester Aβ in the periphery at a symptomatically early stage, thus preferably preventing either the initial stages of, or the severity of, disease progression. Furthermore, prophylactic treatment can be applied to any individual wishing to undertake treatment, regardless of their susceptibility.

In therapeutic applications, the pharmaceutical compositions of this invention are administered to an individual in need thereof; such individuals already suffer from, or are thought to suffer from the disease, pathology, or condition. In general, a dose of an Aβ-binding compound effective for prophylactic treatment or therapy is the same as that for therapeutic treatment or therapy.

EXAMPLES

The following example describes specific aspects of the invention to illustrate the invention and provides a description of the present methods for those of skill in the art. The example should not be construed as limiting the invention, as the examples merely provide specific methodology useful in the understanding and practice of the invention and its various aspects.

Example 1

Transgenic mice that develop AD-related amyloidosis, (i.e., PS/APP mice; See, L. Holcomb et al., 1998, *Nature Med.*, 4(1):97-100) were used for the studies described in this Example to assess how peripheral sequestration of Aβ affected brain Aβ levels. The ganglioside GM1 was utilized as an exemplary Aβ-binding compound, since GM1 is known to bind Aβ strongly, and does not appear to enter the brain. In addition, a second compound, gelsolin, which is too large to cross the blood/brain barrier (BBB), and is completely unrelated to GM1, but which is also known to bind

Aβ with great avidity, was administered peripherally to confirm the universality of the mechanism.

PS/APP mice were injected every two days for two weeks either with GM1 (number of mice =6) (15 mg/kg, ip,), with gelsolin (number of mice =3) (60 μg/kg, ip), or with vehicle, (phosphate buffered saline), (number =7), into the periphery at 9 weeks of age, an age when amyloid pathology in the brain is not visible. The mice were left for 1 week without injections (the "wash-out" period) and were then sacrificed at 12 weeks of age, an age when amyloid deposition has been initiated and measurable levels of Aβ are present in the vehicle treated controls.

The levels of Aβ in the peripheral blood were tested at three time points during the drug administration period (i.e., after 1 week of injections; after two weeks of injections; and after the wash-out period). Data for the third time point only are shown. The levels of Aβ peptides (Aβ40 or Aβ42) in the brain and plasma were assessed by ELISA assay. All of the Aβ in the brain (including Aβ in plaques) was extracted in 70% formic acid (FA). The levels of Aβ peptides in GM1 or gelsolin treated mice were compared with those of vehicle treated controls.

The results presented in Table 1 show that there was a statistically significant ($p<0.05$) decrease in both Aβ40 and Aββẞ42 in the FA-soluble brain fraction in GM1-treated mice compared with those in control animals. This correlates with a statistically significant increase in peripheral Aβ40 and Aβ42 at the same time point. For gelsolin, there was also a significant decrease in Aβ42, which correlated with a statistically significant increase in peripheral Aβ, thus confirming that the general principle of Aβ sequestration in the periphery being linked to reduction of Aβ in the CNS

holds true for very different types of compounds that have the unifying property of being able to bind Aβ in the blood.

Table 1

Changes of brain Abeta load [fmole/mg protein] in GM1- and Gelsolin-treated mice compared with controls (vehicle)

Treatment	n	Abeta40	Abeta42
Vehicle	7	3630 ± 30	4100 ± 200
GM1	6	2147 ± 226	2990 ± 410
	%	59	73
P-value	*p*	0.005**	0.031*
Gelsolin	3	3473 ± 673	2473 ± 167
	%	96	60
P-value	*p*	0.502	0.021*

*: results statistically significant to the 5% level (p=0.05);
**: results statistically significant to the 1% level (p=0.001).

The results presented in Table 2 show the changes in plasma load of both Aβ40 and Aβ42 in GM1-treated mice compared with control (vehicle) animals. As can be observed, there was a significant increase in plasma Abeta levels. For GM1, both Abeta40 and Abeta42 are increased; for gelsolin, Abeta42 is increased to a greater extent than is Abeta40. Thus, the effect with gelsolin may reflect a different preference for Abeta42 over Abeta40.

Table 2

Changes of plasma Abeta load [fmole/ml plasma] in GM1 treated mice compared with controls (vehicle)

Treatment	n	Abeta40	Abeta42
Vehicle	7	100 ± 21	100 ± 7
GM1	6	177 ± 29	124 ± 11
	p	0.021**	0.036*
Gelsolin	3	218 ± 87	153 ± 35
	p	0.016**	0.039*

*: results statistically significant to the 5% level (p=0.05);
**: results statistically significant to the 1% level (p=0.001).

Neither GM1 nor gelsolin is known to cross into the brain from the periphery to any degree. In addition, as part of the studies related to those described in this example, GM1 was introduced directly into the brain of transgenic mice, but no change in Aβ levels was observed. Thus, the results indicate that the effects of GM1 administration in the test mice is due to the sequestration of Aβ in the periphery, thereby leading to a change in dynamics between brain and peripheral Aβ transport. This is the first time that such a result has been shown for a peripherally administered compound that is not an antibody. As such, the invention affords a significant advantage to the art by describing and promoting Aβ-binding compounds that require neither penetration of the brain nor the evocation of an immune response, which are potentially harmful and ineffective ways to modulate the risk of AD in human patients.

It will be appreciated that the use of GM1 or gelsolin in the present example is not limiting to the types of compounds considered to be suitable for use in the present invention. Indeed, in accordance with this invention, any Aβ binding molecule can have the same effect following peripheral administration, thus providing a powerful treatment and therapeutic for AD sufferers, as well as those afflicted with other amyloidoses, e.g., amyloid angiopathy.

Example 2

In this Example, as in Example 1, transgenic mice that develop AD-related amyloidosis, (i.e., PS/APP mice; See, L. Holcomb et al., 1998, *Nature Med.*, 4(1):97-100) were used to assess the Aβ-binding compound chrysamine G (CG) in the peripheral sequestration of Aβ according to this invention, and to determine how the peripheral sequestration of Aβ by this compound affected brain Aβ levels. CG is known to bind Aβ strongly, and is less brain permeable than GM1.

PS/APP mice at 10 weeks of age were injected once either with CG (number of mice=3, dosage: 20 mg/kg) or vehicle (phosphate buffered saline, number of mice=2) into the blood stream. Blood samples were collected prior to treatment (injection) and post-treatment at 10 minutes, 2.5 hours, 5 hours and 25 hours after injection. Blood Aβ levels were compared between pre-treatment versus post-treatment at 10 minutes, 2.5, 5 and 25 hours after injection. The levels of Aβ peptides (Aβ40 or Aβ42) in the plasma were assessed by ELISA immunoassay. The levels of Aβ peptides in the periphery, i.e., plasma, of CG treated mice were compared with plasma Aβ levels in pre-treatment mice at various time points.

Changes in plasma Aβ levels after injection with CG were compared with pre-treatment plasma Aβ levels as shown in Table 3.

Table 3

Changes in plasma Aβ levels after injection with CG

Hours after injection	Aβ42 level [% of pre-treatment time point]
2.5	110 ± 8 (p=0.232)
5	125 ± 8 (p=0.049)
7.5	125 ± 7 (p=0.034)
24	112 ± 13 (p=0.341)
48	111 ± 10 (p=0.256)

The results presented in Table 3 show that were was a statistically significant ($p<0.05$) increase in Aβ, as represented by Aβ42 determination, in the plasma of the mice injected with CG at 5 and 7.5 hours after injection. Changes in plasma load of Aβ42 after injection with CG were compared to the plasma Aβ level at pre-treatment time points. As can be observed, there was a significant increase in plasma Aβ levels after injection of CG.

Following a one-week wash out period, the effect in brain Aβ level after continuous injection was examined. PS/APP mice were injected every day for one week into the periphery, with either CG (number of mice = 3) (20 mg/kg, ip,) or vehicle (phosphate buffered saline) (number of mice = 2), at 11 weeks of age, an age when amyloid pathology in the brain is not visible. The mice were sacrificed at 12 weeks of age, an age when amyloid deposition has been initiated and measurable levels of Aβ are present in the vehicle treated controls.

The levels of Aβ in the peripheral blood were tested at the end of the administration period. The levels of Aβ peptides (e.g., Aβ40 or Aβ42) in the brain and plasma were assessed by ELISA assay. All of the Aβ in the brain (including Aβ in plaques) was extracted in 70% formic acid (FA). The levels of Aβ peptides in CG treated mice were compared with those of vehicle treated controls. The results presented in Tables 4 and 5 show that there was a statistically significant ($p<0.05$) decrease in Aβ40 and/or Aβ42 in the FA-soluble brain fraction in CG-treated mice compared with those in control animals tested 1 week after injection. This correlates with a statistically significant increase in peripheral Aβ40 and Aβ42 at the same time point.

Table 4

Change of plasma Aβ levels 1 week following injection with CG

	Plasma Aβ42 level [% of control]
Vehicle	100 ± 9
CG treated	331 ± 10
	(p=0.0081)

Table 5

Change of brain Aβ 1week following injection with CG

	Aβ40 [fmol/ml]	Aβ42 [fmol/ml]
Vehicle	926 ± 26	1209 ± 292
CG	541 ± 50	540 ± 271
	(*P*=0.0031)	(*P*=0.2038)*

*: Because of data variation, Aβ42 was not statistically significant, p=0.20.

The method of the present invention for determining elevated levels of Aβ in the periphery for the purposes of diagnosing, screening, or monitoring patient treatment, treatment outcome, or the course and/or severity of amyloid-related disease in an individual preferably involves a pretreatment or baseline value for assessing peripheral elevation of Aβ levels in the individual undergoing testing. In the examples presented herein, an elevation of plasma Abeta was compared by percentage pre-treatment time point of an individual animal. Similar comparative assessments of pretreatment and treatment Abeta levels can be employed for the testing of other mammals, including humans, particularly because the range of Abeta levels can be large between and among individuals. As a nonlimiting guide, a representative non-elevated level of Abeta in human plasma (e.g., periphery) is about 25%, as determined experimentally (e.g., Mehta et al., 2000, *Ibid.*).

The contents of all patents, patent applications, published PCT applications and articles, books, references, reference manuals and abstracts cited herein are hereby incorporated by reference in their entirety to more fully describe the state of the art to which the invention pertains.

As various changes can be made in the above-described subject matter without departing from the scope and spirit of the present invention, it is intended that all subject matter contained in the above description, or defined in the appended claims, be interpreted as descriptive

and illustrative of the present invention. Many modifications and variations of the present invention are possible in light of the above teachings.

WHAT IS CLAIMED IS:

1. A method of treating amyloid beta (Aβ)-associated disease, comprising administering an amyloid beta (Aβ)-binding agent in the periphery of an individual in need thereof, wherein said agent binds to Aβ in the periphery, sequesters Aβ in the periphery and concomitantly decreases Aβ levels in the brain of the individual undergoing treatment, in the absence of immunomodulating agents.

2. The method according to claim 1, wherein the amyloid beta (Aβ)-associated disease is selected from Alzheimer's disease, β-amyloid related problems of Down's syndrome, vascular dementia (cerebral amyloid angiopathy) and amyloidosis.

3. The method according to claim 1, wherein the amyloid beta (Aβ)-binding agent is selected from the group consisting of GM1 ganglioside, gelsolin, an Aβ imaging agent, a β-sheet breaker, a β-sheet formation inhibitor and a derivative of an amyloid beta (Aβ)-staining dye.

4. The method according to claim 1, wherein the derivative of the amyloid beta (Aβ)-staining dye is 1,4-bis(3-carboxy-4-hydroxyphenylethenyl)-benzene and 5,5'-{(1,1' biphenyl)-4, 4'-diylbis(azo)} bis {2-hydroxybenzoic acid} disodium salt (chrysamine-G).

5. The method according to claim 1, wherein amyloid beta (Aβ)-binding agent is virtually brain impermeable.

6. A method for sequestering Aβ in the periphery comprising blood or blood components of an individual in need thereof, comprising:

a) administering an agent having binding affinity for amyloid beta (Aβ) in the periphery of the individual in need thereof;

b) sequestering Aβ in the periphery, thereby concomitantly decreasing Aβ levels in the brain of the individual.

7. The method according to claim 6, wherein the agent having binding affinity for amyloid beta (Aβ) is administered in the absence of immunomodulating agents or brain penetrance.

8. The method according to claim 6, wherein the individual is suffering from an amyloid beta (Aβ)-associated disease.

9. The method according to claim 8, wherein the amyloid beta (Aβ)-associated disease is selected from Alzheimer's disease, β-amyloid related problems of Down's syndrome, vascular dementia (cerebral amyloid angiopathy) and amyloidosis.

10. The method according to claim 6, wherein the agent having binding affinity for amyloid beta (Aβ) is selected from the group consisting of GM1 ganglioside, gelsolin, an Aβ imaging agent, a β-sheet breaker, a β-sheet formation inhibitor and a derivative of an amyloid beta (Aβ)-staining dye.

11. The method according to claim 10, wherein the derivative of the amyloid beta (Aβ)-staining dye is 1,4-bis(3-carboxy-4-hydroxyphenylethenyl)-benzene and 5,5'-{(1,1' biphenyl)-4, 4'-diylbis(azo)} bis {2-hydroxybenzoic acid} disodium salt (chrysamine-G).

12. A method of monitoring the effectiveness of drug treatment of beta-amyloid related diseases, comprising:

(a) assessing levels of Aβ in the periphery of a recipient of the drug treatment; and

(b) determining an elevation of the levels of Aβ in the periphery of the recipient.

13. The method according to claim 12, wherein the levels (Aβ) are assessed by an assay that detects Aβ.

14. The method according to claim 13, wherein the assay is (i) a radioisotopic immunoassay or (ii) a non-isotopic immunoassay.

15. The method according to claim 14, wherein the non-isotopic immunoassay is selected from a fluorescent immunoassay, a chemiluminescent immunoassay, or an enzymatic immunoassay (ELISA).

16. The method according to claim 12, wherein the drug treatment comprises an agent having binding affinity for amyloid beta (Aβ).

17. The method according to claim 16, wherein the agent is selected from the group consisting of GM1 ganglioside, gelsolin, an Aβ imaging agent, a β-sheet breaker, a β-sheet formation inhibitor and a derivative of an amyloid beta (Aβ)-staining dye.

18. The method according to claim 17, wherein the derivative of the amyloid beta (Aβ)-staining dye is 1,4-bis(3-carboxy-4-hydroxyphenylethenyl)-benzene and 5,5'-{(1,1' biphenyl)-4, 4'-diylbis(azo)} bis {2-hydroxybenzoic acid} disodium salt (chrysamine-G).

19. The method according to claim 12, wherein the monitoring occurs at about 1-25 days following administration of drug to the recipient.

20. The method according to claim 12, wherein the monitoring occurs at about 5-10 days following administration of drug to the recipient.

21. A method of treating amyloidosis in a subject, said method comprising administering in the periphery of said subject an amyloid

beta (Aβ) binding agent, said agent selected from the group consisting of GM1 ganglioside, gelsolin, an Aβ imaging agent, a β-sheet breaker, a β-sheet formation inhibitor and a derivative of an amyloid beta (Aβ)-staining dye, for a time and under conditions suitable for the agent to bind amyloid beta (Aβ), sequester amyloid beta (Aβ) in the periphery and decrease amyloid beta (Aβ) levels in the brain of the individual.

22. The method according to claim 21, wherein the derivative of the amyloid beta (Aβ)-staining dye is 1,4-bis(3-carboxy-4-hydroxyphenylethenyl)-benzene and 5,5'-{(1,1' biphenyl)-4, 4'-diylbis(azo)} bis {2-hydroxybenzoic acid} disodium salt (chrysamine-G).

Serum insulin-like growth factor I regulates brain amyloid-β levels

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Levels of insulin-like growth factor I (IGF-I), a neuroprotective hormone, decrease in serum during aging, whereas amyloid-β (Aβ), which is involved in the pathogenesis of Alzheimer disease, accumulates in the brain. High brain Aβ levels are found at an early age in mutant mice with low circulating IGF-I, and Aβ burden can be reduced in aging rats by increasing serum IGF-I. This opposing relationship between serum IGF-I and brain Aβ levels reflects the ability of IGF-I to induce clearance of brain Aβ, probably by enhancing transport of Aβ carrier proteins such as albumin and transthyretin into the brain . This effect is antagonized by tumor necrosis factor-α, a pro-inflammatory cytokine putatively involved in dementia and aging. Because IGF-I treatment of mice overexpressing mutant amyloid markedly reduces their brain Aβ burden, we consider that circulating IGF-I is a physiological regulator of brain amyloid levels with therapeutic potential.

Insulin and insulin-like growth factor I (IGF-I) are structurally related circulating hormones. Although the role of insulin as a critical metabolic hormone has been known for decades, the biological significance of IGF-I is still uncertain. Classically, IGF-I was considered the major mediator of the actions of growth hormone on peripheral tissues[1]. However, recent evidence in mutant mice with low levels of serum IGF-I has challenged this view because the mice grow normally[2]. A somewhat unexpected role for this circulating growth factor on brain function is supported by recent observations. For instance, serum IGF-I is required for many of the effects of physical exercise on the brain, including neuroprotection[3–5]. Moreover, systemic IGF-I modulates adult neurogenesis and increases neuronal excitability[3,5,6]. Thus, circulating IGF-I is a physiologically relevant neuroprotective factor with a wide spectrum of actions in the adult brain.

Additional evidence in support of this idea comes from the observations that many neurodegenerative conditions show altered levels of serum IGF-I, and that levels of serum and brain IGF-I decrease with age—a well established risk factor for neurodegenerative diseases[7–9]. For example, both insulin and IGF-I have been related to the pathogenesis of Alzheimer disease (AD) because affected patients show changes in these circulating hormones and an abnormal response to insulin, probably related to inhibition of insulin binding[10–13]. Furthermore, insulin modulates cellular clearance of amyloid-β (Aβ)[14] and IGF-I protects neurons against its toxic effects[15]. It has been argued that the major factor leading to increased brain levels of soluble amyloid-β in AD patients is increased tissue accumulation rather than overproduction[16]. Thus, impaired Aβ clearance may be an important etiopathogenic factor in this devastating disease. However, the processes involved in Aβ clearance from brain tissue are still poorly understood, although a relevant role is assigned to transport mechanisms at the blood–brain barriers (BBB).

The BBB interfaces in the vascular endothelium and choroid plexus (CP) epithelial lining have been classically considered as anatomical and functional barriers for blood-borne macromolecules[17]. Nevertheless, several serum proteins that can bind soluble Aβ penetrate into the brain from the bloodstream by receptor-mediated transcytosis[18,19]. Because IGF-I receptors are abundantly expressed in the cells making up the barriers[20], blood-borne IGF-I could potentially affect barrier permeability to Aβ carrier proteins. Therefore, we have explored the role played by IGF-I in brain Aβ clearance and the possible involvement of IGF-I in transport processes at the BBB interface.

Serum IGF-I modulates brain Aβ clearance

To determine whether serum IGF-I influences brain Aβ under a biologically relevant condition, we used aging rats (>18-mo-old) that have increased levels of Aβ in the hippocampus[21] and compared them with young rats (Fig. 1*a*). IGF-I in aging rats was raised in blood and brain through chronic subcutaneous infusion (50 μg/kg)[22]. We used western blotting and ELISA to determine Aβ levels in the CP, cerebrospinal fluid (CSF) and forebrain. After IGF-I treatment, Aβ levels in the hippocampus (Fig. 1*a*) and cortex (data not shown) of aging rats were reduced to those seen in young rats. Aβ levels in the CSF of aged rats were increased by IGF-I (Fig. 1*b*). In the CP the opposite effect was seen; older rats showed high levels of Aβ as compared with 3-month-old rats, and treatment with IGF-I restored Aβ levels back to those seen in young rats (Fig. 1*c*). Gliosis associated with aging[23] was also eradicated by IGF-I treatment. Reactive astrocytes expressing GFAP and vimentin are abundant in the cerebral cortex (Fig. 1*d*) and hippocampus (data not shown) of aging rats. However, reactive astrocytes are scarce in IGF-I-treated rats (Fig. 1*d*). Immunoblot quantification of GFAP levels in the cerebral cortex (Fig. 1*d*) and hippocampus (data not shown) confirmed a marked reduction of this protein after treatment with IGF-I.

An opposing relationship between levels of circulating IGF-I and brain Aβ may have a pathophysiological impact. Indeed, transgenic mice with a 60% decrease in serum IGF-I levels as a result of liver-specific deletion of the IGF-I gene[2] (LID mice, which have 35 ± 3 ng/ml serum IGF-I versus 82 ± 7 ng/ml in control littermates) have prematurely increased brain levels of Aβ (Fig. 1*e* and *f*). After one year, Aβ levels in the hippocampus and in the cortex of LID mice are significantly higher than in controls (Fig. 1*e*

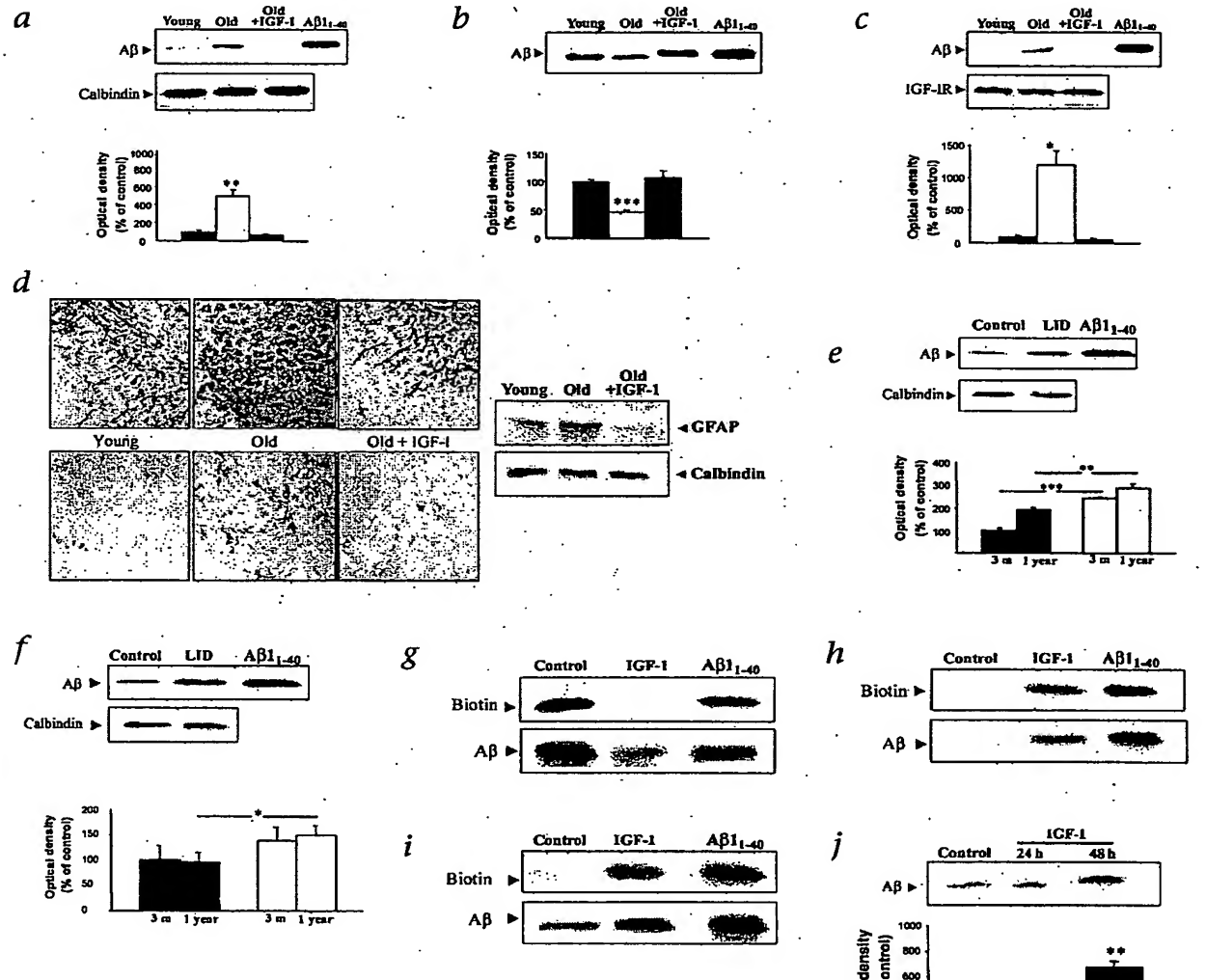

Fig. 1 IGF-I modulates brain Aβ levels. *a–c*, Aβ levels in the hippocampus (*a*), CSF (*b*) and CP (*c*) of aging rats are restored to those found in young animals after IGF-I treatment. Representative western blots of $Aβ_{1-40}$ and corresponding densitometric measurements in the 3 brain compartments of young (■), aging (□) and aging IGF-I-treated (■) rats ($n = 6$ per group). *, $P < 0.05$; **, $P < 0.01$ and ***, $P < 0.001$ versus young animals. $Aβ_{1-40}$ (1 μg) was run as a marker in every gel. Samples were re-blotted with control antibodies to assess equal protein load per lane. *d*, Gliosis associated with aging[23], as determined by antibody staining for GFAP (top) and vimentin (bottom) in brain frontal cortex astrocytes was ameliorated by IGF-I. Right gel, High levels of GFAP immunoreactivity in the cortex of aging rats were reduced by IGF-I (calbindin levels are shown for comparison). *e* and *f*, LID mice (□) have increased Aβ in the hippocampus (*e*) and cortex (*f*) at early age, as compared with control littermates (■). Significant increases were seen in both areas in 1-y-old mice ($n = 4$ per group). *, $P < 0.05$; **, $P < 0.01$ and ***, $P < 0.001$ versus respective ages in control littermates. Representative blots of 1-y-old brains are shown. *g–l*, Intraparenchymal injection of $bAβ_{1-40}$ to young adult rats results in its accumulation in the cortex (*g*). Systemic administration of IGF-I before $bAβ_{1-40}$ injection results in disappearance of $bAβ_{1-40}$ from the cortex and its appearance in CSF (*h*) and CP (*i*). Upper gels, Blot with streptavidin to reveal bAβ; lower gels, anti-$Aβ_{1-40}$ was used to reveal total $Aβ_{1-40}$. $bAβ_{1-40}$ was run in parallel in each gel as a control. *j*, I.c. injection of IGF-I increases endogenous Aβ levels in CSF in a time-dependent fashion ($n = 4$). *, $P < 0.05$ and **, $P < 0.001$ versus saline-injected rats.

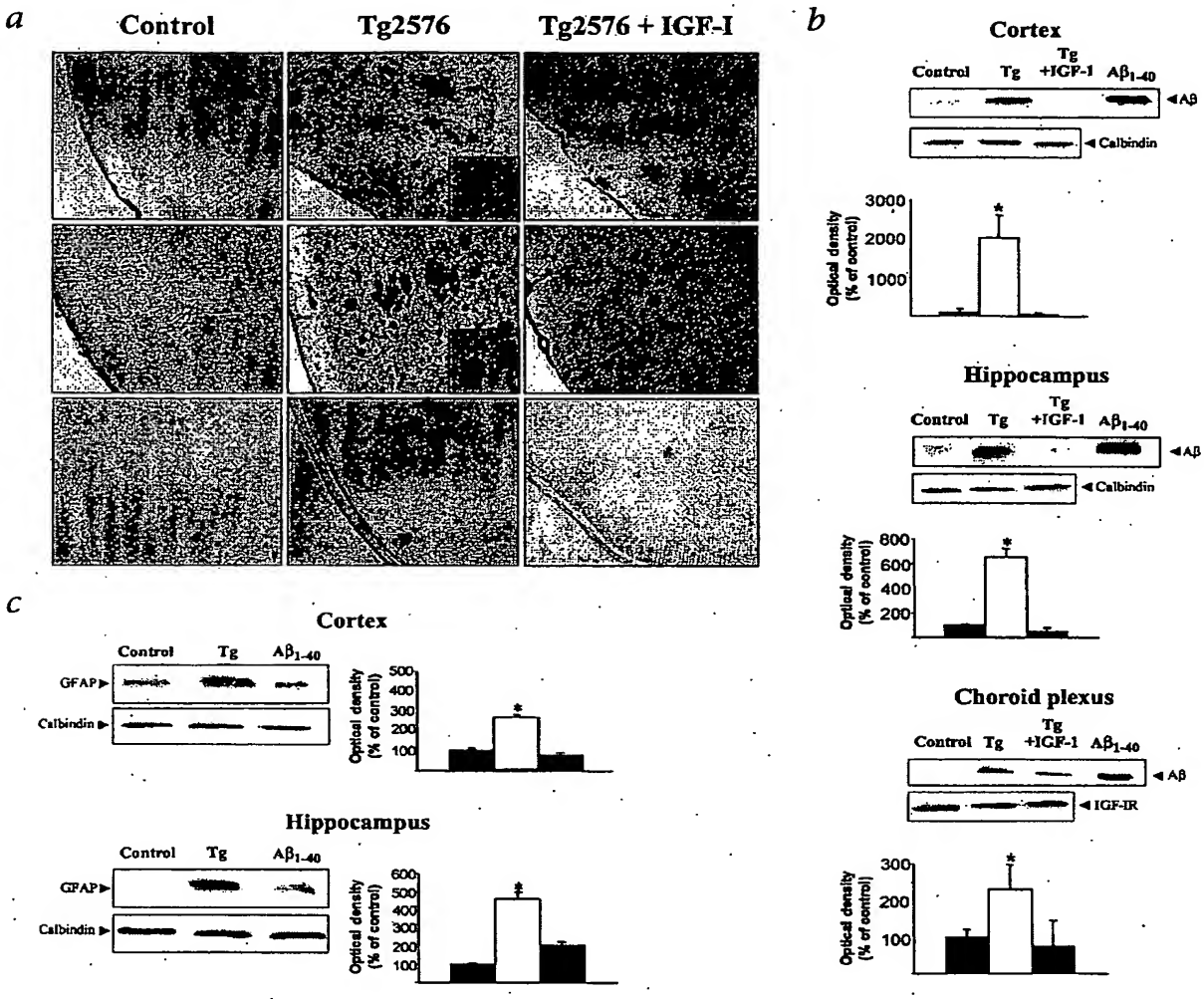

Fig. 2 IGF-I decreases brain Aβ burden in a model of AD amyloidosis. *a*, Amyloid deposits in pyriform cortex of g2576 mice are reduced by s.c. IGF-I treatment. Human Aβ in transgenic mice was detected with a human-specific antibody (6E10, top panels). Murine and human Aβ were detected with an antibody that recognizes both human and murine Aβ (middle). Amyloid deposits were revealed with Congo Red staining (bottom). Insets, Similar Aβ deposits were detected regardless of the antibody used. Note absence of staining in control littermates. Magnification: large panels, ×50; insets, ×1,000. *b*, Western-blot evaluation of Aβ levels in the cortex, hippocampus and CP confirms that Aβ is significantly reduced by IGF-I treatment in Tg2576 mice. *c*, High GFAP levels (gliosis) associated with brain amyloidosis are corrected by IGF-I both in the cortex and the hippocampus of Tg2576 mice (n = 3–9 per group). ■, control; □, transgenic; ■, transgenic + IGF-I. *, $P < 0.05$ versus control littermates.

and *f*). A possible explanation is that IGF-I modulates brain Aβ clearance; to test this, we injected biotinylated $Aβ_{1-40}$ ($bAβ_{1-40}$) in the cerebral cortex of young adult rats. Two days before Aβ injection (60 nM), a group of rats had received an intracarotid (i.c.) injection of IGF-I (10 μg per rat) that results in a 3-fold increase in IGF-I CSF levels[3], or a saline control. One hour after injection, biotinylated $Aβ_{1-40}$ was present at high levels in the cortex of saline-injected rats but not detectable in IGF-I-injected rats (Fig. 1*g*). On the contrary, $bAβ_{1-40}$ can be detected in the CP (Fig. 1*h*) and CSF (Fig. 1*i*) one hour after brain injection only in animals pretreated with IGF-I. To confirm that IGF-I increases clearance of Aβ, we studied its effect on endogenous Aβ levels in the CSF. Notably, i.c. IGF-I increases CSF Aβ levels in a time-dependent manner after the injection (Fig. 1*j*).

To exploit the ability of IGF-I to lower brain Aβ levels, we tested it in a mouse model of AD amyloidosis. Tg2576 mice overexpressing a mutant form of human amyloid precursor protein (APP695) show high brain levels of Aβ peptides together with cognitive decline[24]. Furthermore, like AD patients affected with this mutation[11], Tg2576 mice have lower serum IGF-I levels (57.6 ± 4 ng/ml in transgenics versus 78.4 ± 7 ng/ml in non-transgenic controls; $P < 0.05$), which makes them well suited for IGF-I therapy. After one month of treatment with IGF-I, SDS-soluble brain Aβ levels as well as Aβ staining in the brain parenchyma of 1-y-old Tg2576 mice

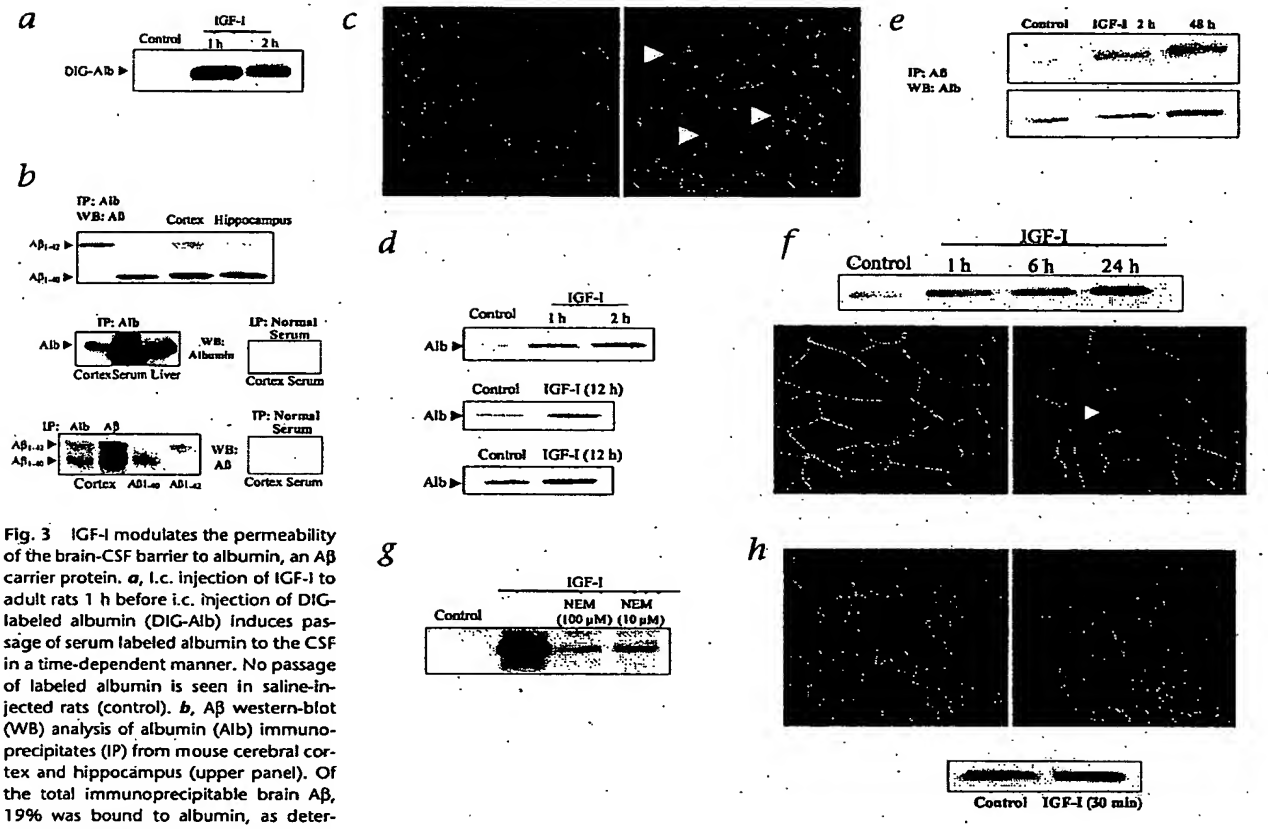

Fig. 3 IGF-I modulates the permeability of the brain-CSF barrier to albumin, an Aβ carrier protein. *a*, I.c. injection of IGF-I to adult rats 1 h before i.c. injection of DIG-labeled albumin (DIG-Alb) induces passage of serum labeled albumin to the CSF in a time-dependent manner. No passage of labeled albumin is seen in saline-injected rats (control). *b*, Aβ western-blot (WB) analysis of albumin (Alb) immunoprecipitates (IP) from mouse cerebral cortex and hippocampus (upper panel). Of the total immunoprecipitable brain Aβ, 19% was bound to albumin, as determined by western blot to detect Aβ in albumin or Aβ immunoprecipitates from mouse cortex (lower panel). Control experiments show the specificity of the anti-albumin (middle panel) and anti-Aβ (lower panel) immunoprecipitates for their respective antigens. A similar (middle panel) or 10-fold (lower panel) higher concentration of normal serum IgGs from non-immunized rabbits fails to detect antigen. A liver extract was run as a positive control for albumin. Synthetic $Aβ_{1-40}$ and $Aβ_{1-42}$ (1 μg) were run in parallel lanes. *c*, Choroid plexus cells (identified with synaptophysin, green) accumulate labeled albumin (red) after i.c. injection of IGF-I (right panel) but not after saline (left). Arrowheads indicate albumin-stained CP cells. *d*, Levels of endogenous albumin are upregulated in the CSF (top), CP (middle), and brain cortex (bottom) of IGF-I-injected rats in a time-dependent manner ($n = 3$). *e*, Albumin western-blot analysis of Aβ immunoprecipitates from CSF (upper panel) and serum (lower panel) obtained from young adult rats injected i.c. with IGF-I or saline (control). 48 h after IGF-I injection, the amount of albumin bound to Aβ is increased. *f*, *In vitro* transcytosis of DIG-albumin through a CP epithelial monolayer is markedly potentiated by IGF-I. Addition of IGF-I to the upper chamber of the CP cultures together with DIG-albumin induces accumulation of labeled albumin in the lower chamber in a time-dependent manner. As seen *in vivo*, cultured CP epithelial cells accumulate DIG-albumin upon exposure to IGF-I (lower photomicrographs). Synaptophysin-positive epithelial cells (green) stain with anti-digoxigenin antibodies only after IGF-I (red). Left, control; right, IGF-1. Arrowhead indicates digoxigenin-labeled cell. *g*, Accumulation after IGF-I of DIG-albumin in the lower chamber of cultured CP cells is inhibited in a dose-dependent manner by NEM. *h*, Exposure of CP monolayers to IGF-I does not alter perimembrane distribution or Tyr-phosphorylation of the tight-junction protein ZO-1. Left, control; right, IGF-1. Gel shows western blot with anti-pTyr of ZO-1 immunoprecipitates.

were significantly reduced (Fig. 2*a* and *b*), whereas levels of Aβ in the CP tended to normalize (Fig. 2*b*). Stereological analysis of brain amyloid stained with an antibody that recognizes both endogenous (murine) and transgenic (human) Aβ peptides (MBL) (Fig. 2*a*) indicated that treatment with IGF-I reduces the percentage of brain parenchyma stained with amyloid. Although no Aβ staining was found in non-transgenic mice ($n = 6$), amyloid staining in Tg2576 mice treated with IGF-I ($n = 5$) showed a 49% decrease in hippocampus (CA1 and dentate gyrus; 3.5 ± 0.2% in untreated Tg2576 mice ($n = 4$) versus 1.8 ± 0.4% in IGF-I-treated, $P < 0.05$), a 60% decrease in parietal cortex (15.7 ± 0.6% in untreated transgenics versus 6.3 ± 0.6% in treated, $P < 0.0001$) and a 30% decrease in the pyriform cortex (10.0 ± 3.4% in untreated versus 7.0 ± 3.0% in treated mice, $P = 0.05$). Further analysis with a human-specific antibody against Aβ (E610) (Fig. 2*a*) gave qualitatively similar results: a 37% decrease in amyloid staining in the pyriform cortex (19.1 ± 0.9% in saline-treated Tg2576 mice versus 12.2 ± 0.8% in IGF-I-treated, $P < 0.05$). We also used Congo Red to detect amyloid deposits and found fewer and smaller deposits after IGF-I treatment (Fig. 2*a*). In addition, forebrain gliosis in Tg2576 was decreased after IGF-I as high levels of GFAP in the cortex and hippocampus were significantly reduced (Fig. 2*c*).

To better quantify changes in $Aβ_{1-40}$ and $Aβ_{1-42}$ after IGF-I, we measured them by ELISA in additional groups of animals. Confirming the results obtained with the immunoblots, aging rats treated with IGF-I had a 90% decrease in brain $Aβ_{1-40}$: 134 ± 127

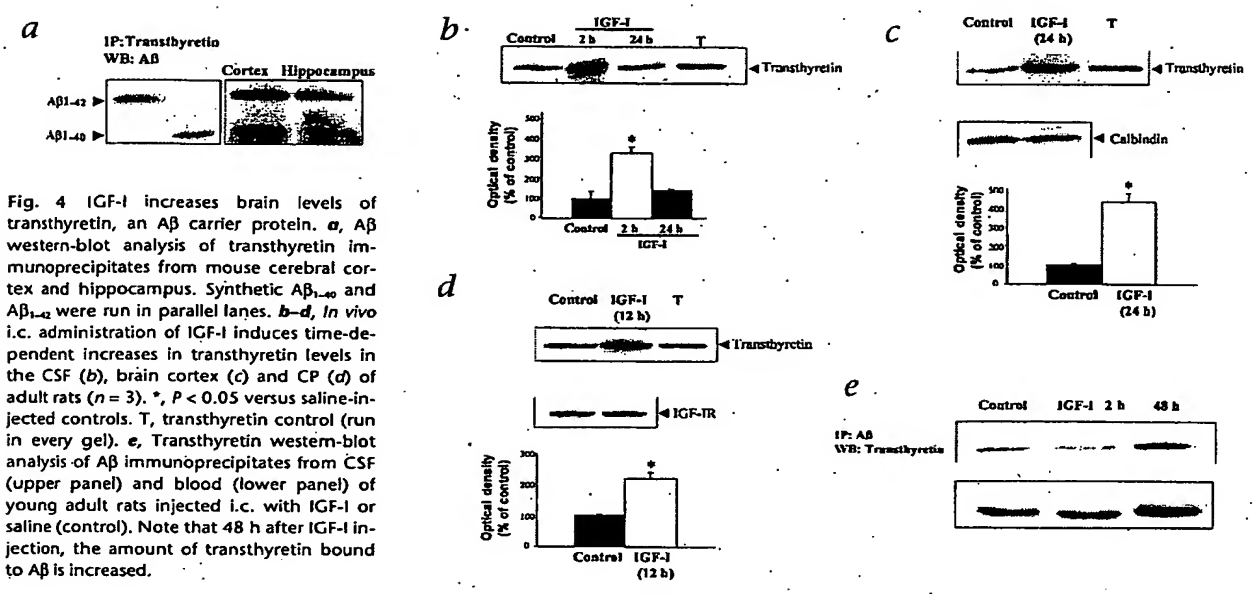

Fig. 4 IGF-I increases brain levels of transthyretin, an Aβ carrier protein. *a*, Aβ western-blot analysis of transthyretin immunoprecipitates from mouse cerebral cortex and hippocampus. Synthetic $Aβ_{1-40}$ and $Aβ_{1-42}$ were run in parallel lanes. *b–d*, *In vivo* i.c. administration of IGF-I induces time-dependent increases in transthyretin levels in the CSF (*b*), brain cortex (*c*) and CP (*d*) of adult rats ($n = 3$). *, $P < 0.05$ versus saline-injected controls. T, transthyretin control (run in every gel). *e*, Transthyretin western-blot analysis of Aβ immunoprecipitates from CSF (upper panel) and blood (lower panel) of young adult rats injected i.c. with IGF-I or saline (control). Note that 48 h after IGF-I injection, the amount of transthyretin bound to Aβ is increased.

pmol/g of hippocampus in aged IGF-I-treated rats ($n = 5$) versus 1,131 ± 1,151 in untreated aged rats ($n = 5$), (young rats ($n = 4$) had 118 ± 74 pmol/g) and a 99% decrease in brain $Aβ_{1-42}$ (10.9 ± 4 pmol/g in IGF-I-treated versus 1,181 ± 1,335 in untreated rats, $P < 0.05$) (young controls had 127 ± 98 pmol/g). On the contrary, as was also seen by immunoblotting, CSF $Aβ_{1-40}$ and $Aβ_{1-42}$ were increased after IGF-I treatment. Levels of CSF $Aβ_{1-40}$ were 1,920 ± 121 pM in IGF-I-treated versus 626 ± 71 in untreated aged rats, $P = 0.05$; young controls had 2,209 ± 109 pM. Levels of CSF $Aβ_{1-42}$ were 950 ± 102 pM in treated versus 539 ± 69 in untreated; young controls had 1,088 ± 97 pM. Similarly, Tg2576 mice treated with IGF-I had an 89% reduction in human $Aβ_{1-40}$ in their hippocampus: 187 ± 182 pmol/g in IGF-I-treated Tg2576 mice ($n = 4$) versus 1,705 ± 255 in untreated mutants ($P < 0.05$). IGF-I-treated Tg2576 mice also had a 100% decrease in human $Aβ_{1-42}$: undetectable levels in IGF-I-treated mice versus 1,194 ± 41 in untreated Tg2576 mice ($P < 0.001$). Control littermates ($n = 5$) had undetectable levels of human $Aβ_{1-40}$ and $Aβ_{1-42}$. In addition, IGF-I-deficient mice show a marked increase in both brain Aβ forms; levels of $Aβ_{1-40}$ were 919 ± 164 pmol/g in LID mice ($n = 4$) versus 128 ± 12 in control littermates ($n = 5$, $P = 0.01$). Levels of $Aβ_{1-42}$ were 593 ± 125 pmol/g in LID mice versus 87 ± 20 in controls ($P < 0.05$).

IGF-I modulates CP permeability to Aβ carrier proteins

While exploring the effects of IGF-I on the permeability of the blood-CP barrier we found that transport of labeled albumin from the bloodstream into the CSF was greatly enhanced by systemic injection of IGF-I (10 μg per rat, 1 h before albumin injection) (Fig. 3*a*). Changes in the permeability of the barrier to serum proteins may underlie increased Aβ clearance by IGF-I, because serum proteins such as albumin might transport Aβ peptides[25]. Indeed, brain albumin co-immunoprecipitated with endogenous Aβ (Fig. 3*b*). Of the total brain Aβ that could be immunoprecipitated, 19% was bound to albumin (Fig. 3*b*). This agrees with recent findings indicating that albumin binds to Aβ *in vitro*[13]. We therefore analyzed the mechanisms underlying IGF-I actions on albumin transport across the CP. Rats receiving a systemic injection of digoxigenin-labeled albumin (10 μg) 1 hour after IGF-I accumulated it in CP cells (Fig. 3*c*). To determine the physiological implications of this observation, we assessed whether transport of endogenous serum albumin across the CP is affected by IGF-I. Indeed, albumin levels increased in the CSF and CP after systemic IGF-I administration (Fig. 3*d*). This leads to accumulation of albumin in brain cortex at later times (Fig. 3*d*). Significantly, increased entrance of albumin after IGF-I is paralleled by increased levels of Aβ bound to albumin

Table 1 Serum IGF-I levels regulate brain levels of albumin and transthyretin

Animal model	Area	Albumin (% control)	Transthyretin (% control)
Aged rat	CSF	9.4 ± 3.5**	17 ± 2.6***
+ IGF-I		123 ± 37.5	92.3 ± 10.8
	Choroid plexus	17.9 ± 2.2**	35 ± 3.5**
		100.9 ± 7.2	89.6 ± 11
	Hippocampus	30.6 ± 6.2**	35 ± 3.5*
		71.5 ± 8.3	117.4 ± 32.2
Tg2576 mouse[b]	Cortex	0.9 ± 0.5**	22.7 ± 2.7*
+ IGF-I		59.7 ± 2.9*	85.5 ± 24.6
	Choroid plexus	28.7 ± 1.6*	23.8 ± 2.8*
		97.7 ± 9.6	92 ± 20
	Hippocampus	44.2 ± 1.8*	56.4 ± 9.3*
		80.7 ± 5.6	96.7 ± 10
LID mouse[c]	Cortex	46.2 ± 5.6**	37.7 ± 4.35***
	Choroid plexus	45 ± 4**	27.6 ± 5.6***
	Hippocampus	60.3 ± 8.9*	62.6 ± 3.6**

Data are expressed as mean ± s.e.m. [a], Controls are 3-mo-old rats; $n = 6$. [b], Controls are non-transgenic littermates; $n = 3–9$. [c], Controls are $Lox^{+/+}$ littermates; $n = 4$. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$

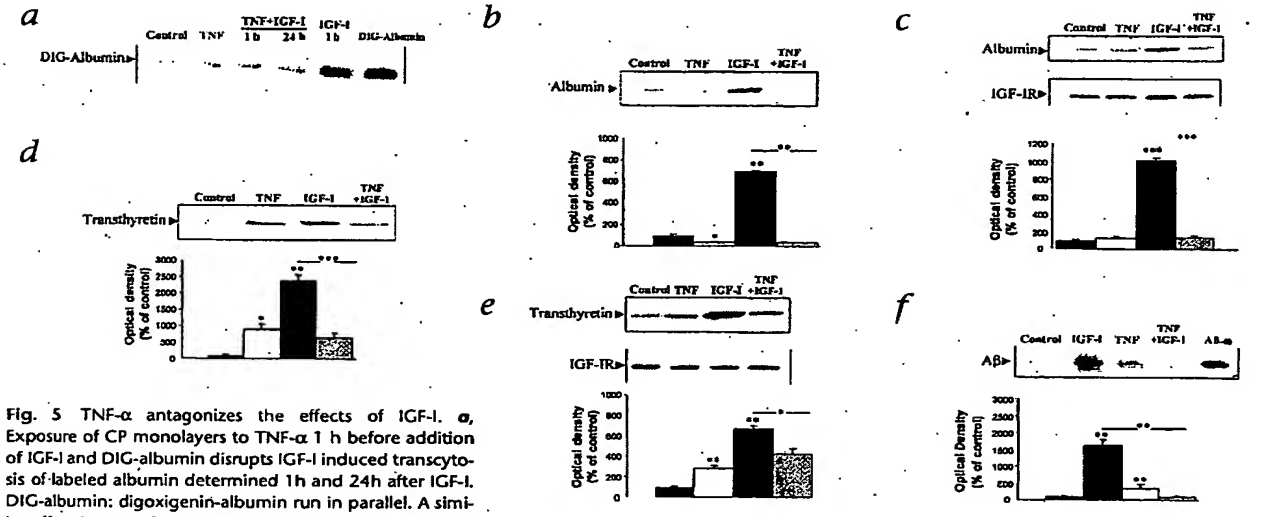

Fig. 5 TNF-α antagonizes the effects of IGF-I. *a*, Exposure of CP monolayers to TNF-α 1 h before addition of IGF-I and DIG-albumin disrupts IGF-I induced transcytosis of labeled albumin determined 1h and 24h after IGF-I. DIG-albumin: digoxigenin-albumin run in parallel. A similar effect is seen after *in vivo* i.c. injection of TNF-α given 1 h before IGF-I ($n = 4$). *b* and *c*, TNF-α abrogated the IGF-I-induced increase in CSF (*b*) and CP (*c*) albumin determined 1 h later. *d* and *e*, Similarly, transthyretin levels in CSF (*d*) and CP (*e*) did not increase after simultaneous i.c. injection of TNF-α and IGF-I ($n = 4$). *f*, Stimulation of brain Aβ clearance by i.c. IGF-I, as determined by increased Aβ levels in the CSF, is blocked by administration of TNF-α ($n = 4$). $A\beta_{1-40}$: run in parallel. $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0,001$ versus controls, or as indicated. In *b*–*f*: ■, control; □, TNF; ■, IGF-1; ▨, TNF+IGF-1.

both in the CSF and in blood (Fig. 3*e*). This indicates that IGF-I favors the entrance of albumin into the brain. In turn, increased availability of albumin may enhance transport of brain Aβ to the blood stream through its binding to albumin.

We examined in further detail the IGF-I-induced transport of albumin across CP cells using a double-chamber culture system that mimics the blood-CSF interface. Choroid plexus monolayers transcytosed albumin (1 μg/ml) in response to IGF-I (1×10^{-7} M), as indicated by cytosolic labeling of the cells and accumulation of labeled albumin in the lower culture chamber (Fig. 3*f*). This process is dependent on endocytosis as it is blocked by the endocytosis blocker *N*-ethyl maleimide (NEM) (10–100 μM) (Fig. 3*g*). The effect is specific because the permeability of the CP monolayer to inulin, which uses a paracellular route, or glucose, which uses a transport-specific system across the cells, are not affected by IGF-I (data not shown). IGF-I did not affect the functional and anatomical integrity of the epithelial barrier because neither the peri-plasma membrane localization of the tight junction–associated protein ZO-1 nor its rate of tyrosine phosphorylation are affected by IGF-I (Fig. 3*h*).

Albumin is transcytosed across epithelia through the multiligand complex megalin–cubilin that participates in transport of other serum proteins probably involved in brain Aβ clearance such as transthyretin[19,26,27]. As with albumin, endogenous Aβ co-immunoprecipitates with transthyretin (Fig. 4*a*). Whereas albumin preferentially bound to $A\beta_{1-40}$ (Fig. 3*b*), transthyretin mostly bound to $A\beta_{1-42}$ (Fig. 4*a*). We explored whether IGF-I modulates brain levels of transthyretin. IGF-I (i.c.) increased levels of transthyretin in the CP, CSF and brain cortex of adult rats (Fig. 4*b*–*d*). Increased brain levels of transthyretin after IGF-I paralleled increased Aβ bound to transthyretin in CSF and in blood (Fig. 4*e*). The latter finding suggests that transthyretin is also involved in IGF-I-induced transport of Aβ out of the brain. In agreement with these observations, we found that reduced Aβ burden after IGF-I treatment of either aging rats, mice overexpressing mutant APP or LID mice is paralleled by increased brain levels of albumin and transthyretin (Table 1).

TNF-α antagonizes the effects of IGF-I

To further analyze the modulation of brain Aβ by IGF-I we explored the role of tumor necrosis factor-α (TNF-α). We focused on TNF-α for two reasons. First, this pro-inflammatory cytokine blocks IGF-I effects on several target cells, including neurons[28]. Second, increased levels of TNF-α (and other pro-inflammatory cytokines) have been suggested to participate in age- and neurodegeneration-associated brain impairment[29,30]. Indeed, serum levels of TNF-α in aging rats and Tg2576 mice were 40% and 124% higher than those of young rats and control littermates, respectively: 316 ± 130 pg/ml of TNF-α in 18-month-old rats versus 225 ± 95 pg/ml in 3-month-old rats, and 1041 ± 480 pg/ml in Tg2576 mice versus 463 ± 132 pg/ml in controls.

We found that TNF-α (10 ng/ml) blocked IGF-I-induced passage of labeled albumin through a CP monolayer (Fig. 5*a*). More notably, i.c. administration of TNF-α (500 ng per rat) 1 hour before IGF-I blocked passage of albumin through the CP barrier, as determined by decreased levels of albumin in the CSF and the CP of adult rats (Fig. 5*b* and *c*). TNF-α (i.c.) also counteracted the effects of systemic IGF-I on transport and production of transthyretin by the CP (Fig. 5*d*), resulting in lower levels of tranthyretin in the CSF (Fig. 5*e*). These results were paralleled by inhibition of the IGF-I-induced accumulation of soluble Aβ in the CSF at later times (48 h after IGF-I), when TNF-α is given 2 hours before IGF-I (Fig. 5*f*). Therefore, TNF-α blocks the stimulatory effects of IGF-I on Aβ clearance.

Discussion

Our findings indicate that serum IGF-I modulates brain levels of Aβ, a protein of unknown biological function but related to the pathogenesis of AD dementia[16]. IGF-I also modulated brain availability of albumin and transthyretin, two proteins that bind Aβ. Upon systemic IGF-I administration, brain levels of these two proteins increased and the amount of Aβ bound to them increased in the CSF and in the blood. In addition, in all experimental models

tested, higher brain levels of tranthyretin and albumin after IGF-I administration correlated with higher Aβ in the CSF and lower Aβ in the brain. By contrast, low serum IGF-I was linked to premature brain amyloidosis and low brain levels of transthyretin and albumin. Furthermore, TNF-α antagonism of IGF-I-mediated enhancement of barrier permeability to albumin and transthyretin paralleled its blockade of the beneficial effects of IGF-I on brain Aβ burden. Therefore, IGF-I may reduce brain Aβ load, at least in part, by enhancing its clearance through carrier proteins such as albumin and transthyretin.

These data suggest that age-related brain amyloidosis is linked to low serum IGF-I (ref. 8). In addition, high TNF-α levels during aging and in AD (refs. 29,30 and present observations) may also contribute to amyloidosis. TNF-α participates in the inflammatory process associated with AD (ref. 30), disrupts brain-barrier properties[31] and antagonizes other actions of IGF-I in the brain[28]. Therefore, accumulation of brain Aβ in aging and dementia may involve impaired Aβ clearance stemming from interference of TNF-α with the actions of IGF-I at the level of the BBB. Since IGF-I is beneficial in age- and AD-related brain disturbances[15,32,33], we suggest that serum IGF-I is involved in physiological and pathological brain aging. Notably, the IGF-I/insulin pathway seems related to aging very early in phylogeny[34].

Although the ability of IGF-I to regulate albumin and transthyretin passage through the CP may be relevant to several aspects of CP physiology, we will focus on its relationship to neuroprotection. The CP epithelium expresses the membrane complex cubilin–megalin that translocates into the CSF several serum proteins able to transport Aβ (refs. 26,27,35). Albumin crosses the CP barrier and accumulates in the CSF and brain parenchyma under pathological conditions. This may possibly explain the neuroprotective role of albumin in the injured brain[36]. Nevertheless, we suggest that increased albumin passage induced by IGF-I does not reflect a dysfunction of the barrier properties of the CP epithelium as in pathological circumstances such as inflammation[25]. In support of this, IGF-I did not produce a generalized increase in epithelial permeability, as indicated by lack of passage of inulin or glucose, and reduced passage of leptin (data not shown). Moreover, our *in vitro* experiments suggest that epithelial tight-junction stability, which accounts for the barrier properties of the CP epithelium[17], was not compromised by IGF-I. Hence, it is possible that regulation of CP transcytotic albumin passage by serum IGF-I is important for the neuroprotective actions of IGF-I. For example, low albumin levels in brains of aged rats or Tg2576 mice may be related to the observed decrease in serum IGF-I and consequently, to reduced Aβ clearance. Thus, IGF-I may be a physiological modulator of the passage of albumin into the brain, under normal as well as pathological circumstances.

Transthyretin is a wide-spectrum carrier protein involved in retinol, thyroid hormone and Aβ transport[18]. Mutations in this protein underlie vascular amyloidosis[37], a metabolic disease leading to polyneuropathy and vascular derangements. CP cells not only allow the transport of liver-derived transthyretin from the blood into the brain, but also secrete large amounts of this protein[25,38]. To our knowledge, there is no known modulatory mechanism accounting for regulated secretion/transcytosis of transthyretin by the CP. Our present findings provide a possible modulatory signal: circulating IGF-I. Although our observations link IGF-I regulation of brain transthyretin levels to brain Aβ load, it is likely that other biological correlates of the actions of transthyretin, such as brain levels of thyroid hormone[39], will also be affected by serum IGF-I. This requires further study.

In summary, serum IGF-I increases clearance of Aβ in the brain and upregulates brain levels of Aβ carriers such as albumin and transthyretin. Of note, not only AD patients, but also Down syndrome patients that develop premature brain amyloidosis, have low serum albumin and transthyretin levels[40]. In view of the numerous protective actions exerted by IGF-I in the brain, we cannot eliminate albumin and transthyretin-independent offects of IGF-I leading to increased Aβ clearance. Possible effects include increasing Aβ transport at the cellular level, as recently reported for insulin[14] or modulating Aβ-degrading proteases; the latter process suggested to be central in pathological accumulation of Aβ (ref. 16). Regardless of the mechanisms involved, we propose that administration of IGF-I may be of therapeutic value in treatment and prevention of brain amyloidosis in humans.

Methods

In vivo treatments. Adult (3-mo-old) and aging (>18-mo-old) Wistar rats were from our inbred colony. LID mice were generated using the Cre-Lox system under the control of the albumin promoter[2]. LID mice have decreased serum IGF-I levels but normal postnatal growth[2]. $Lox^{+/+}$ (not expressing Cre-recombinase) littermates were used as controls. The Tg2576 line overexpressing human APP695 containing the double mutation K670N, M671L under the control of the prion protein promoter[24] was used as a model of AD amyloidosis. These mice show AD-type neuropathology in an age-dependent fashion[24]. At 1 year, Tg2576 mice show elevated levels of $Aβ_{1-40}$, the major soluble form of brain amyloid. Age-matched non-transgenic littermates were used as controls. IGF-I (GroPep, Adelaide, Australia) was administered either acutely as a bolus injection into the carotid artery (10 μg/rat)[3] or chronically using minipumps (Alzet 2004 in rats and 1002 in mice, at 50 μg/kg, equivalent to 12 μg/rat/day; Alzet, Cupertino, California), as described[3]. TNF-α (500 ng/rat, PreProTech, London, UK) was injected through the carotid 1 h before IGF-I. Digoxigenin-labeled albumin was injected i.c. (10 μg/rat) 1 h after IGF-I. Albumin was labeled with digoxigenin-3-*O*-methylcarbonyl-aminocaproic-acid-*N*-hydroxy-succinimide ester (Boehringer, Germany) as described[3]. Biotinylated-$Aβ_{1-40}$ (60 nM, 2 μl) was injected in the cerebral cortex of 3-mo-old rats (stereotaxic coordinates: 0.9 anterior from bregma, 1.9 lateral and 2.5 mm ventral[41]). $Aβ_{1-40}$ (Calbiochem, San Diego, California) was biotinylated with EZLink-Biotin (Pierce, Rockford, Illinois) following the manufacturer's instructions. Control mice received saline. CSF samples (150 μl) were collected under anesthesia from the cisterna magna. Mice were then perfused transcardially with saline or paraformaldehyde for biochemical and immunocytochemical analysis, respectively. All animal procedures followed regulatory guidelines of the Spanish Ministry of Agriculture as determined by EU directive 86/609/EEC.

Chorold plexus cultures. Epithelial cell monolayers were prepared as described[42]. Choroid plexuses were dissected, enzymatically digested and filtered. Cells were collected and plated in a two-chamber culture well (Becton-Dickinson, Franklin Lakes, New Jersey) with insulin (5 μg/ml), transferrin (5 μg/ml), sodium selenite (5 ng/ml), epidermal growth factor (10 ng/ml), basic fibroblastic growth factor (5 ng/ml, Boehringer), hypoxanthine (500 μM) and 10% FBS (Gibco, Barcelona, Spain). Plates were coated with collagen (Becton Dickinson) and 10 μg/ml laminin. Barrier properties of the monolayer were determined by its electrical resistance and permeability characteristics. The resistance of a chamber membrane without cells was ~20 Ω while that of the epithelial monolayer was 150 Ω, similar to that seen *in vivo*[42]. Permeability was assessed by a diffusion assay using [^{14}C]-carboxylated inulin (Amersham, Buckinghamshire, UK) as a paracellular marker and [^{14}C]-1-D-glucose (Amersham) as a transcellular marker. Inulin, which cannot cross the tight-junction barrier, shows negligible diffusion (0.94% of the coefficient of permeability of a cell-free culture well), whereas glucose, which has a regulated transcytotic passage, shows a 12.5% coefficient of permeability. For the experiments, cells were grown to confluence for 5–7 d, serum-starved for 4 h, and DIG-albumin (1 μg/ml), IGF-I (1×10^{-7} M) or TNF-α (10 ng/ml, 1 h before IGF-I) was added to the upper chamber. Culture medium in the lower chamber was collected at various time points. Cells were either fixed for immunocytochemical assay or homogenized for immunoblot determination.

Immunoassays. Western-blot analysis and immunoprecipitation were performed as described[43]. Samples were solubilized in NP-40-containing and then 2% SDS-containing buffers, electrophoresed and blotted. A rabbit antibody against Aβ (MBL, Nagoya, Japan) that recognizes rodent and human N-terminal Aβ forms was used for immunoprecipitations. Streptavidin-peroxidase was used to reveal biotinylated-Aβ and anti-digoxigenin for digoxigenin-labeled albumin. Membranes were stripped and reblotted with either anti-calbindin (Swant, Bellinzona, Switzerland) or antibodies to IGF-I receptors[43] to assess protein loading. Immunohistochemistry and stereological analyses were done as described[5]. For detection of Aβ deposits, brain sections were pre-incubated in 88% formic acid. To determine Aβ burden in Tg2576 mice, we also used a human-specific antibody to Aβ (6E10; Sigma). Results are expressed as percentage of brain area covered with amyloid. Amyloid deposits in parenchyma were also evaluated with Congo Red staining. Aβ sandwich ELISAs were performed as described[44]. For detection of human Aβ, we used the 6E10 antibody in the first layer and anti-$Aβ_{1-40}$ or anti-$Aβ_{1-42}$ (Calbiochem) in the top layer. For rodent Aβ, we used the 4G8 antibody (Sigma), which recognizes both rodent and human Aβ, in the first layer and the same antibodies against $Aβ_{1-40}$ and $Aβ_{1-42}$, respectively, in the top layer. To quantify both soluble and insoluble forms of Aβ, samples were extracted with formic acid and centrifuged at high speed, and supernatants were assayed, as described[45]. A TNF-α ELISA was performed in serum samples following the manufacturer's instructions (Chemicon, Temecula, California). Antibodies used were monoclonal anti-digoxigenin (Boehringer), anti-sinaptophysin, anti-GFAP, anti-Vimentin (Boehringer), anti-pTyr (Transduction Labs, Lexington, Kentucky) and polyclonal anti-ZO-1 (Santa Cruz, Santa Cruz, California), anti-albumin (Bethyl, Montgomery, Texas), and anti-transthyretin (Santa Cruz); all were diluted 1:500–1:1000. Secondary antibodies used were Alexa-coupled (Molecular Probes, Eugene, Oregon). Non-immune normal rabbit serum was used as a control for immunoprecipitation studies.

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Competing interests statement

The authors declare competing financial interests: see the website (http://www.nature.com/naturemedicine) for details.

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Alzheimer's soluble amyloid β is a normal component of human urine

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Abstract Soluble Aβ (Saβ) is normally present at a low concentration in human plasma and cerebrospinal fluid. Although the factors involved in the regulation of Saβ plasma levels are still unknown, we have explored its excretion in the urine as one of the possible homeostatic mechanisms. The presence of Saβ in the urine was investigated via immunoprecipitation experiments with anti-Aβ antibodies followed by detection and identification by immunoblot, MALDI mass spectrometry and sequence analysis. Soluble Aβ (4.3 kDa) immunoreactivity was present in the urine of normal donors, Down's syndrome individuals as well as in patients with renal disorders exhibiting glomerular or mixed proteinuria. Edman degradation of the immunoprecipitated material yielded the intact Aβ N-terminus and mass spectra analysis indicated the existence of a major component at *m/z* 4327, corresponding to the molecular mass of Aβ1-40. Semiquantitative data obtained from the immunoprecipitation experiments indicate that under normal conditions the daily excretion of intact Saβ in the urine represents less than 1% of the circulating pool.

Key words: Alzheimer's disease; Down's syndrome; Proteinuria; Saβ

1. Introduction

Amyloid beta (Aβ) is the major constituent of the fibrils deposited into senile plaques and cerebral blood vessels of patients with Alzheimer's disease (AD), Down's syndrome (DS), Hereditary Cerebral Hemorrhage with Amyloidosis of Dutch origin and normal aging (reviewed in [1]). Although originally thought to be an aberrant degradation product of its precursor molecule βPP, this peptide is now known to be a normal soluble component (Saβ) present at very low concentration in plasma and cerebrospinal fluid (CSF) [2–5]. The major form of Saβ, Aβ1-40, is homologous to the amyloid protein extracted from cerebrovascular lesions, although minor Saβ species with heterogeneity and length similar to the Aβ components deposited in senile plaques were also found in circulation [5,6]. Soluble Aβ was also described as a normal component in brain parenchyma, from where it can be extracted in aqueous solutions. Interestingly, when soluble fractions of AD and DS brain homogenates were compared with control brains, an increased amount of Saβ was detected [7,8]. This increase appears to precede the appearance of Aβ deposits [9], suggesting that Saβ species may indeed represent immediate precursors of the deposited fibrils. However, the origin of the Aβ deposited in the brain is still debatable; it may be locally synthesized, it may come from the circulation, or a combination of both sources.

Soluble Aβ circulates in plasma associated mainly with lipoprotein particles, specifically high-density lipoproteins (HDL), co-localizing with apolipoproteins J (apoJ), E (apoE) and A-I (apoA-I) [10]. All these apolipoproteins exhibit high binding affinity for Aβ peptides [11–13]. ApoJ, in particular, displays a very high-affinity interaction for non-aggregated forms of the Aβ peptides and has been shown to maintain and stabilize the peptide solubility in vitro [14]. Several lines of evidence indicate that the blood-brain barrier (BBB) has the capability to regulate the brain uptake and clearance of Saβ. For the brain uptake, at least two different receptor-transport mechanisms have been identified. One involves the peptide when it is complexed to apoJ, a proposed carrier molecule for circulating Saβ species. A 36-fold higher uptake than that of the extracellular space marker sucrose was found for the complex Aβ-apoJ in the guinea pig vascular-brain perfusion model. The receptor implicated in the BBB transport was identified as gp330 or megalin, the receptor for apoJ [15]. The other receptor transport mechanism pertains to the uptake of free Aβ peptides and was studied in guinea-pigs [16,17], squirrel monkeys [18], mice [19] and rats [20]. The uptake of the free peptide, although one-fourth of that of the complex Aβ-apoJ, is saturable and specific in the guinea-pig brain perfusion model, compromising cell surface molecules that are not yet fully identified. One possible candidate is the receptor for advance glycation-end products (RAGE), a recently described receptor for Aβ species [21].

The BBB also appears to regulate the clearance of brain Saβ species. Infusion of ^{125}I-labeled Aβ1-40 into one lateral ventricle in the rat brain has shown that in as little as 3.5 min 30% of the peptide was cleared from ventricular CSF. Ten minutes after infusion ~70% of the radioactivity disappeared from the brain and was recovered in the blood, liver, kidney and urine [22]. In order to clarify to which extent the urinary excretion of Saβ might be an important mechanism for Saβ clearance from the circulation, we have investigated its presence in urine from normal donors, in specimens from DS patients with normal levels of urinary proteins as well as in samples from individuals with different degree of glomerular or mixed glomerular-tubular proteinuria. Down's syndrome urines were studied since it is known that plasma Saβ levels are elevated in these patients [23]. The methodology used herein, immunoprecipitation, immunoblot analysis, matrix-as-

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sisted laser desorption/ionization mass spectrometry (MALDI-MS) and N-terminal sequence clearly identified the presence of Aβ1-40 as the major Saβ specie of human urine.

2. Materials and methods

2.1. Samples

Twenty-four-hour urine specimens were collected from two normal donors (proteinuria < 5 mg/dl), three patients with glomerular proteinuria (210–600 mg/dl), five patients with mixed glomerular-tubular proteinuria (7–420 mg/dl) and seven cases of DS (proteinuria < 5 mg/dl). DS urines were kindly provided by Dr. K. Wisniewski (Institute for Basic Research at Staten Island) from well-characterized patients who had been subjected to chromosomal analysis. All samples were dialyzed against distilled water using 1000 Da cut-off membrane and lyophilized prior to use.

2.2. Immunoprecipitation

Fifty microliters of paramagnetic Dynabeads M-450 coated with goat anti-mouse IgG (Dynal) were allowed to interact for 3 h at room temperature with a mixture of 3 μl of monoclonal antibody 6E10 (anti-Aβ 1-17; Senetek) and 3 μl of monoclonal antibody 4G8 (anti-Aβ17-24; Senetek). After incubation, unbound antibody was removed by washing the beads with 10 mM phosphate (Ph 7.4) containing 150 mM NaCl (PBS) and 0.1% bovine serum albumin. For the immunoprecipitation experiments, 5 mg of urinary proteins were solubilized in 1 ml of PBS, added to the tube containing the paramagnetic beads coated with anti-Aβ antibodies and incubated overnight at 4°C. After the incubation, the beads were washed 3 times with PBS, resuspended in Laemmli sample buffer and loaded onto the SDS-polyacrylamide gel. Alternatively, the beads were eluted with 10 μl of isopropylic alcohol/water/formic acid (4:4:1) mixture and analyzed by MALDI-MS.

2.3. Immunoblot analysis

Immunoprecipitated samples were separated on Tris-Tricine 16% SDS-PAGE and electroblotted onto nitrocellulose membrane (Bio-Rad) using 3-cyclohexylamino-1-propanesulphonic acid (pH 11) containing 10% methanol. Membranes were blocked for 1 h with 5% low-fat milk in PBS containing 0.05% Tween-20 and incubated overnight with monoclonal 6E10 (1:500), followed by horseradish peroxidase-labeled goat anti-mouse $F(ab')_2$ (1:2000; Amersham). Immunoblots were visualized with an enhanced chemiluminescence (ECL) detection kit and exposed to Hyperfilm ECL (Amersham). The intensity of the bands was semi-quantitatively evaluated using a Umax Power-Look scanner and the NIH Image 1.60 software.

2.4. Sequence analysis

Twenty milligrams of urinary proteins from two cases of mixed glomerular-tubular proteinuria were separated on Tris-Tricine 16% SDS-PAGE and transferred to polyvinylidene difluoride membranes (Immobilon P, Millipore) using the same conditions described above. After transfer, the membrane was stained with Coomassie Blue and the 4.3 Kda protein bands were excised and sequenced on a 477A protein sequencer (Applied Biosystems).

2.5. MALDI-MS

Soluble Aβ from the urine samples immunoprecipitated as described above was eluted from the beads in 10 μl of water/isopropyl alcohol/formic acid (4:4:1) and subjected to MALDI-MS using the dried droplet method with α-cyano-4-hydroxicynnamic acid solution as a matrix. Samples were processed at the Mass Spectrometry Facility at the Skirball Institute (NYU Medical Center).

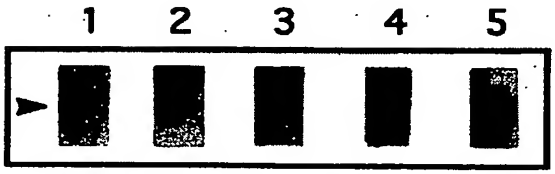

Fig. 1. Western Blot analysis of immunoprecipitated Saβ from urine samples. Fluorograms of the Saβ immunoprecipitated (arrowhead) from 5 mg of urinary proteins from normal donors (lane 1), Down's Syndrome (lane 2) and non-demented individuals with glomerular (lane 3) or mixed proteinuria (lane 4). For comparison, lane 5 displays the immunoreactivity of 10 ng of synthetic peptide Aβ1-40 immunoprecipitated under the same experimental conditions.

3. Results and discussion

In order to identify the presence of Saβ in the urine, we developed an immunoprecipitation method based on the use of paramagnetic beads coated with goat anti-mouse IgG that were coupled to anti-Aβ monoclonal antibodies. Amongst the available antibodies immunoreactive with Aβ we found that the mixture of 6E10 plus 4G8 allowed the highest recoveries and a high sensitivity. Under the conditions tested, immunoprecipitation of 0.2 ng of synthetic Aβ1-40 rendered a clearly visible 4 Kda band in the corresponding fluorograms. Parallel experiments using Protein A/G agarose reached the detection limit at 1.0 ng.

For each immunoprecipitation experiment, 5 mg of urinary samples from either normal donors, patients with glomerular proteinuria, individuals with mixed glomerular-tubular proteinuria, or DS patients were used. As indicated in Fig. 1, immunoblot analysis of the immunoprecipitated material after separation in SDS-PAGE revealed the presence of a specific immunoreactive band of about 4 Kda with identical electrophoretic mobility of that of synthetic Aβ1-40. Soluble Aβ immunoreactivity was present in normal donors (proteinuria < 5 mg/dl) (lane 1), although it was consistently more prominent in DS samples exhibiting a similar degree of proteinuria (< 5 mg/dl) (lane 2). It was also detected in samples from patients with mixed glomerular-tubular proteinuria (7–420 mg/dl) (lane 3) as well as predominant glomerular components (proteinuria 210–600 mg/dl) (lane 4).

Densitometric analysis of the different bands allowed us to estimate the amount of Saβ peptide present in each sample through the comparison with 10 ng of synthetic Aβ1-40 peptide immunoprecipitated under the same conditions (lane 5). For the normal donors tested (proteinuria < 5 mg/dl), the excretion of Saβ was calculated at 0.81 ± 0.26 ng/5 mg of

Table 1
Estimation of mean Saβ levels in urine samples

Pathology	n	Proteinuria (mg/dl)	Saβ (ng/5 mg urinary proteins)	Excretion of Saβ (ng/24 h)
Normal donors	2	< 5	0.81 ± 0.26	13 ± 4
Down's syndrome	7	< 5	2.13 ± 0.70	32 ± 11
Glomerular or mixed proteinuria	8	7–600	1.24 ± 0.32	20–2000

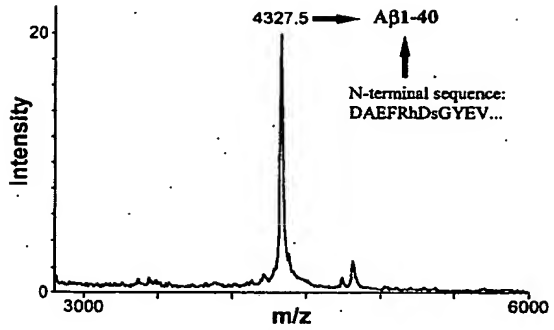

Fig. 2. Mass spectrometry of immunoprecipitated Saβ from urine samples. Aliquots of lyophilized urine samples (5 mg) were immunoprecipitated using monoclonal antibodies 6E10 and 4G8 coupled to paramagnetic beads as described in Section 2. The immunoprecipitated material was eluted using 10 μl of isopropyl alcohol/water/formic acid (4:4:1) mixture, applied on the spectrometer's probe using the dried droplet method in alpha-cyano-4-hydroxycinnamic acid matrix, and analyzed by MALDI-MS. For calibration purposes, synthetic Aβ1-40 was used as standard of mass.

urinary proteins (13 ± 4 ng/24 h). The Down's syndrome patients analyzed (all with proteinuria < 5 mg/dl) excreted a higher amount of Saβ (2.13 ± 0.7 ng/5 mg of urinary proteins, equivalent to 32 ± 11 ng/24 h), which might correlate with their higher plasma concentration [23] as a result of the gene dosage. Taking into consideration that the levels of Saβ in plasma are in the range of 1 ng/ml [4;23] and that under normal conditions the volume of the urine excreted in a 24 h period is around 1–1.5 l, the daily excretion of Saβ in the urine represent less than 1% of the total circulating pool. These values are obviously increased several fold in patients with either glomerular or mixed tubular-glomerular proteinuria; in these cases, where the amount of urinary proteins can reach values of 600 mg/dl or more, the daily excretion of Saβ could reach 2000 ng/24 h. It is possible that when there is loss of permselectivity, Saβ complexes from the circulation could pass through the altered filtration barrier, accounting for the higher excretion of Saβ (Table 1).

The identity of Saβ present in the urine samples was corroborated by N-terminal sequence analysis. The 4.3 Kda bands obtained upon immunoprecipitation of 20 mg of urinary proteins from two cases of mixed glomerular-tubular proteinuria were transferred to a polyvinylidene difluoride membrane and subjected to automatic Edman degradation. The sequences DAEFRxDxGxEV and DAEFRhDsGYEV were obtained, corresponding to the intact N-terminus of Aβ. No N-terminal heterogeneity was found in the samples analyzed. The mass spectrometry analysis of the immunoprecipitated samples showed a main signal at *m/z* 4327, corresponding to the molecular mass of native Aβ1-40 (Fig. 2). In all the cases studied, the major specie identified in urine was the Aβ1-40 in accordance to that specie's predominance in plasma.

The results presented here demonstrate that Saβ is a normal component of human urine. Under normal conditions, where the renal filtration-resorption function is preserved, the amount of intact Saβ excreted in the urine accounts for less than 1% of the total circulating pool, indicating that the majority of the circulating Saβ is catabolized or excreted by a different mechanism. Due to the association of Saβ with HDL particles, it is possible that Saβ catabolic/excretory pathways may follow those of the lipoprotein particles. If this is the case, it would be expected that the liver would be the key organ involved in the catabolism of Saβ and perhaps in the excretion of the peptide or its degradation products in the bile. Whether urinary Saβ levels are altered in sporadic AD or in familial AD associated with either βPP or presenilin mutations remains to be determined. However, in view of our data (Table 1), potential AD diagnostic methods based on the measurement of urine and/or plasma levels of Saβ should simultaneously evaluate the coexistence of pathological conditions that can alter glomerular filtration and/or tubular resorption.

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Characterization of Apolipoprotein J-Alzheimer's Aβ Interaction*

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The main component of Alzheimer's amyloid deposits, Aβ, has been found also as a soluble (sAβ) normal constituent of biological fluids and cell culture supernatants. Whether or not sAβ is the immediate precursor of Aβ, it is clear that peptides with the same amino acid sequence can have both fibrillar and non-fibrillar conformations. The interconversion mechanism from one form to another is presently under intensive investigation. We have previously described that (i) a synthetic peptide $A\beta_{1-40}$ immobilized on affinity matrices was able to retrieve apolipoprotein J (apoJ) from plasma and cerebrospinal fluid; and (ii) the interaction of sAβ with apoJ occurs *in vivo*, as demonstrated by the ability of anti-apoJ to co-precipitate sAβ from normal cerebrospinal fluid. We have characterized the binding between $A\beta_{1-40}$ and apoJ and found that the interaction is saturable, specific, and reversible. The dissociation constant of 2×10^{-9} M is indicative of high affinity binding. The stoichiometry of the reaction is 1:1; apoJ has five times more affinity for fresh $A\beta_{1-40}$ than for the aggregated peptide. Competitive inhibition studies carried out with apolipoprotein E (isoforms E2, E3, and E4), transthyretin, vitronectin, and α_1-antichymotrypsin indicate that the complex apoJ·$A\beta_{1-40}$ cannot be dissociated by any of these competitors at physiologic concentrations. The data strongly suggest that apoJ plays an important role as a carrier protein for sAβ.

Amyloid β (Aβ)[1] peptide (39–44 residues) is the main component of the two major neuropathological lesions present in AD, senile plaques, and cerebrovascular amyloid deposits (1, 2). Although Aβ has high tendency to aggregate and make fibrils, a soluble form has been detected in biological fluids (soluble Aβ, sAβ) (3–5). Whether or not sAβ is the immediate precursor of Aβ, it is clear that the same amino acid sequence can have both fibrillar and non-fibrillar conformations; therefore, the knowledge of the factors that influence its behavior in solution will be a step forward in the understanding of AD pathology. In this regard, the existence of specific components named "desaggrins" was previously suggested based on the fact that $A\beta_{1-40}$ spontaneous fibril formation *in vitro* is inhibited in the presence of CSF (6).

Extensive immunohistochemical studies indicate that other proteins are co-deposited with Aβ in senile plaques. Amyloid P-component, α_1-antichymotrypsin (ACT), apolipoprotein E (apoE), apolipoprotein J (apoJ), complement components, vitronectin (Vn), glycosaminoglycans, and extracellular matrix proteins are among the amyloid-associated proteins described so far (7–15). It is not clear whether they are innocent bystanders or their presence is related to the mechanism of amyloidogenesis. Several lines of investigation favor the latter notion, at least for some of them (*i.e.* amyloid P-component and apoE are present in several types of fibrillar deposits but absent in non-fibrillar accumulations representing pre-amyloid lesions) (16). In addition, the apoE gene on chromosome 19, particularly the apoE allele ε4, has been linked to sporadic and late-onset AD (17). The inheritance of the apoE4 allele is today considered a risk factor for AD (1).

Biochemical studies performed *in vitro* have demonstrated a certain degree of binding affinity between Aβ and different proteins, among them apoJ, apoE, transthyretin (TTR), and ACT (12, 18–23). The interactions have been considered in the range of "high avidity binding," although they were not quantitatively evaluated. Using immobilized synthetic peptides homologous to Aβ ($A\beta_{1-40}$), we have shown previously its binding association with plasma and CSF apolipoproteins J and E; moreover, the presence of the complex apoJ-sAβ was confirmed in CSF, indicating that the interaction takes place *in vivo* (18, 20). We are reporting herein the characterization of the complex formation between apoJ and $A\beta_{1-40}$, in the presence and absence of other amyloid-associated proteins.

MATERIALS AND METHODS

Synthetic Peptides and Proteins—Peptide DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVV ($A\beta_{1-40}$, homologous to residues 672–711 of βPP_{770}) was synthesized at the W. M. Keck Facility at Yale University and characterized as described (18). A 1 mg/ml stock solution of $A\beta_{1-40}$ was prepared in 50% acetonitrile containing 0.1% trifluoroacetic acid, aliquoted, and frozen at −80 °C until use. For aggregation studies, 100 ng of $A\beta_{1-40}$ were diluted in 20 μl of PBS (20 mM phosphate, pH 7.4, containing 150 mM NaCl) and incubated at 37 °C for different periods of time (0–72 h). Incubation was terminated by the addition of SDS sample buffer and 5 min boiling, and the degree of aggregation was determined by Tris-Tricine 10% SDS-PAGE (24).

Human plasma apoJ was purchased from Quidel (San Diego, CA); ACT and TTR were obtained from Calbiochem (La Jolla, CA); Vn was purchased from Chemicon (Temecula, CA). Recombinant apoE isoforms 2, 3, and 4 (apoE2, apoE3, and apoE4) were obtained from PanVera (Madison, WI). In all cases, protein purity was corroborated by SDS-PAGE and NH_2-terminal sequence.

Solid-phase Binding Studies—The interaction apoJ-$A\beta_{1-40}$ was studied by enzyme-linked immunosorbent assay (ELISA) using $A\beta_{1-40}$ and purified apoJ. Polystyrene microtiter plates (Immulon 2, Dynatech Lab.; Chantilly, VA) were coated with freshly prepared $A\beta_{1-40}$ (400 ng/100 μl/well) in 0.1 M $NaHCO_3$, pH 8.6, for 2 h at 37 °C. Under these conditions, 10 ng of $A\beta_{1-40}$ (representing 2.5% of the peptide offered) remained bound to the well, as determined by a modification of Quantigold assay (Diversified Biotech., Boston, MA). After blocking with 1% bovine serum albumin, different concentrations of purified apoJ (0–25 nM; 100 μl/well) in PBS were added to the $A\beta_{1-40}$-coated wells and incubated for 3 h at 37 °C. Bound apoJ was detected with monoclonal IF12 (anti-apoJ α-chain, 1:5000), a generous gift from Dr. N. H. Choi-

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[1] The abbreviations used are: Aβ, amyloid β; AD, Alzheimer's disease; s, soluble; CSF, cerebrospinal fluid; ACT, α_1-antichymotrypsin; Vn, vitronectin; TTR, transthyretin; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay.

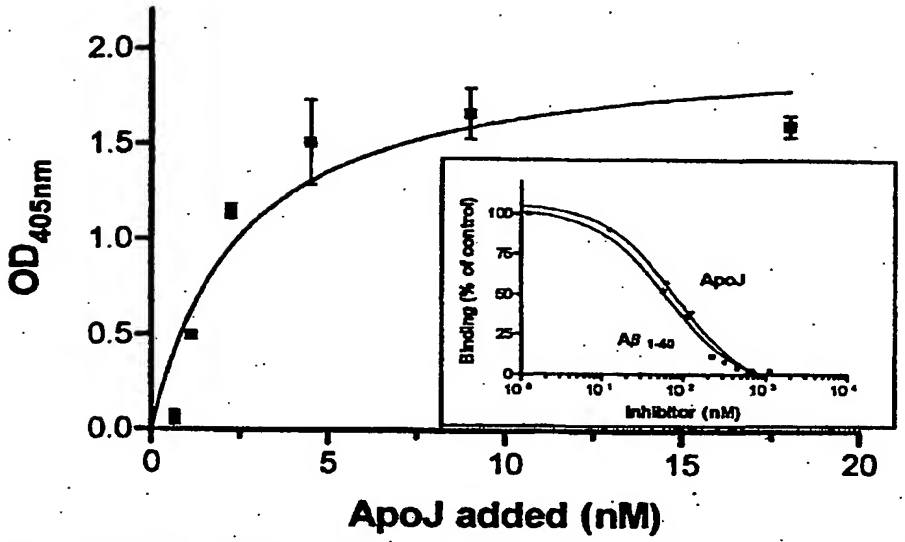

FIG. 1. **Saturation curve for apoJ-Aβ_{1-40} interaction.** Variable concentrations (0–25 nM) of apoJ were incubated with Aβ_{1-40}-coated wells for 3 h at 37 °C. Bound apoJ was detected with monoclonal IF12 and alkaline phosphatase-labeled anti-mouse, as described under "Materials and Methods." Each point represents the mean (±2 S.D.) of five independent duplicate experiments. *Inset*, inhibition of apoJ binding to immobilized Aβ_{1-40}. Increasing concentrations (0–1136 nM) of Aβ_{1-40} were preincubated with apoJ (25 nM) for 3 h at 37 °C. The mixture was added to Aβ_{1-40}-coated wells and incubated for another 3 h at the same temperature. Bound apoJ was determined as described under "Materials and Methods." On separate experiments, native apoJ (0–1136 nM) was coincubated with 25 nM of biotin-labeled apoJ in Aβ_{1-40}-coated wells for 3 h at 37 °C. Bound biotinylated apoJ was detected with alkaline phosphatase-labeled streptavidin. Results are expressed as percentage of binding compared with controls incubated with apoJ alone. Data represent the mean of three independent duplicate experiments. S.D. never exceeded ±6%.

Miura (25), followed by alkaline phosphatase-conjugated goat F(ab')$_2$ anti-mouse IgG (1:3000, BioSource; Camarillo, CA). The reaction was developed for 30 min with *p*-nitrophenyl phosphate in diethanolamine buffer (Bio-Rad), stopped with 0.4 M NaOH, and quantitated at 405 nm on a 7520 Microplate Reader (Cambridge Technology, Watertown, MA). Nonspecific binding was determined using bovine serum albumin-coated wells and/or omitting apoJ in the assay. Binding data were analyzed with the aid of a curve fitting software (GraphPad Prism Version 1.0, GraphPad Software, San Diego, CA).

Inhibition Assays—100 μg of apoJ were biotinylated with Sulfo-NHS biotin (Pierce) according to the manufacturer's specifications; biotin-labeled apoJ was separated from free biotin by chromatography over Sephadex G-10 (Pharmacia) equilibrated in PBS. 0–1136 nM of native unlabeled apoJ was coincubated with 25 nM of biotin-labeled apoJ in Aβ_{1-40}-coated wells (400 ng/well) for 3 h at 37 °C. Bound biotinylated apoJ was detected with alkaline phosphatase labeled streptavidin (1:1000, Amersham) and evaluated as described above.

In separate experiments, variable amounts of fresh Aβ_{1-40} or 24-h self-aggregated Aβ_{1-40} (0–1136 nM) were combined with 25 nM apoJ in PBS and incubated for 3 h at 37 °C. The mixture was then transferred to Aβ_{1-40}-coated wells and incubated for 3 h at 37 °C. Bound apoJ was detected with monoclonal IF12 and alkaline phosphatase-labeled F(ab')$_2$ goat anti-mouse IgG, as described above.

Competitive inhibition assays were performed with apoE2, apoE3, apoE4, ACT, Vn, and TTR. 0–2500 nM of the various competitors in PBS were coincubated with 25 nM of apoJ in PBS in Aβ_{1-40}-coated wells (400 ng/well) at 37 °C for 3 h. Bound apoJ was determined as described above.

Complex Characterization—The apoJ·Aβ_{1-40} complex was prepared by incubation of 5 μg of apoJ with 5 μg of Aβ_{1-40} in PBS for 18 h at 37 °C. The mixture was separated on native 8% non-SDS-PAGE (26), transferred to polyvinylidene difluoride membranes (Immobilon P, Millipore, Milford, MA) using 3-cyclohexylamino-1-propanesulfonic acid pH 11, containing 10% (v/v) methanol, stained with Coomassie Blue, and the protein bands were excised, and sequenced on a 477A protein sequencer (Applied Biosystems, Foster City, CA), as described (18). For immunoblot detection, apoJ·Aβ_{1-40} and apoJ·Aβ_{1-40}(agg) complexes were prepared under identical conditions using 1.1 μmol of apoJ and 1.1 μmol of either fresh or 24-h aggregated peptide. After 18 h of incubation at 37 °C, the complexes were separated by electrophoresis and electroblotted onto Immobilon P as indicated above. Membranes were blocked for 1 h with 5% low-fat dried milk in PBS and incubated overnight with monoclonal 6E10 (anti-Aβ_{1-17}), kindly provided by Dr. K. S. Kim (27), at 1:100 dilution, followed by horseradish peroxidase-labeled sheep anti-mouse F(ab')$_2$ 1:2000 (Amersham). Immunoblots were visualized with an enhanced chemiluminescence (ECL) detection kit and exposed to Hyperfilm ECL (Amersham). The resulting bands were scanned on a PDI densitometer and evaluated with the Quantity One (Version 2.4) software (PDI, Huntington Station, NY).

RESULTS

The interaction between apoJ and Aβ_{1-40} was characterized by means of solid-phase ELISA experiments. A dose-response relationship that reached saturation was obtained when increasing concentrations of apoJ at pH 7.4 were allowed to interact with a constant amount of immobilized Aβ_{1-40} (Fig. 1). Non-linear regression analysis of the specific binding data fitted to a rectangular hyperbola and allowed the calculation of the corresponding dissociation constant K_d of 2 nM. The specificity and reversibility of the interaction were assessed at an apoJ concentration of 25 nM (at which 100% saturation of the Aβ_{1-40}-coated plates was achieved) by means of inhibition experiments with either Aβ_{1-40} or apoJ. When a constant concentration of apoJ in PBS was preincubated with increasing concentrations of freshly prepared Aβ_{1-40} before the addition to Aβ_{1-40}-coated wells, apoJ-binding to immobilized Aβ_{1-40} followed a one-site competition curve (Fig. 1, *inset*). The calculated value for half-maximal inhibition (IC_{50}) was 63 nM. In a separate set of experiments, the binding of biotin-labeled apoJ to immobilized Aβ_{1-40} was competitively inhibited by increasing concentrations of native apoJ. The remaining bound biotinylated apoJ was quantitated with alkaline phosphatase-labeled streptavidin. The data fitted into a one-site competition curve with IC_{50} = 77 nM (Fig. 1, *inset*).

The formation of the apoJ·Aβ_{1-40} complex was visualized by Coomassie Blue staining after electrophoresis on non-denaturing polyacrylamide gels. As shown in Fig. 2, native apoJ exhibits two molecular forms in this non-SDS system: a monomeric component of ~80 kDa and a dimeric form of ~160 kDa (*lane*

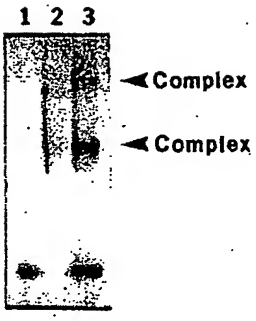

FIG. 2. **Identification and characterization of apoJ-$A\beta_{1-40}$ complexes under non-denaturing conditions.** ApoJ (5 μg) and $A\beta_{1-40}$ (5 μg) were incubated at 37 °C for 18 h and the resultant complexes separated on 8% non-SDS-PAGE, transferred to Immobilon P, and stained with Coomassie Blue. The apparent molecular masses were calculated from the Ferguson plots constructed with known molecular mass standards (α-lactalbumin, 14,200 Da; carbonic anhydrase, 29,000 Da; chicken egg albumin, 45,000 Da; bovine serum albumin, 66,000 Da monomer and 132,000 Da dimer; urease, 272,000 Da monomer and 545,000 Da dimer). The complexes (*arrowheads*) were excised from the membrane and their NH_2-terminal sequence determined. *Lane 1*, $A\beta_{1-40}$; *lane 2*, apoJ; *lane 3*, apoJ-$A\beta_{1-40}$ complex.

2). When complexed to $A\beta_{1-40}$, both components shifted their electrophoretic mobility toward higher molecular masses, resulting in ~85 and 170 kDa bands (an increase of ~5 and 10 kDa, respectively). Amino-terminal sequence analysis of these 85- and 170-kDa components rendered the sequences DQTVSDNELQEMSNQ, SLMPFSPYEPLNFH, and DAEFRHDSGYEVHHQ corresponding to the first 15 residues of the apoJ α-chain, apoJ β-chain, and $A\beta_{1-40}$, respectively. Recovery calculations performed for the first 10 steps of the sequence indicated a 1 to 1 stoichiometry (Table I).

The influence of the degree of $A\beta_{1-40}$ aggregation in its ability to form a complex with apoJ was tested using fresh and aggregated peptide; the resulting complex was visualized via immunoblot analysis after SDS-PAGE using anti-$A\beta_{1-17}$ (monoclonal 6E10), as indicated in Fig. 3. Fresh $A\beta_{1-40}$ exhibited a major monomeric component and a minor dimeric form while the tetrameric aggregates were almost negligible (*lane 1, arrowheads*). A similar aliquot of the synthetic peptide that had been incubated for 24 h at 37 °C showed an increase in the amount of dimers and tetramers in addition to the typical smear-like electrophoretic appearance which indicates the presence of multiple minor components of higher molecular mass (*lane 3*). When fresh $A\beta_{1-40}$ was incubated with apoJ for 18 h at 37 °C (*lane 2*), the presence of the 85-kDa apoJ-$A\beta_{1-40}$ complex was immunodetected by anti-$A\beta_{1-17}$ (*arrow*); the free peptide exhibited the same polymerization pattern as the one shown in *lane 1*. When 24-h-aggregated $A\beta_{1-40}$ was incubated with apoJ under identical conditions, the presence of a less intense 85-kDa complex was detected by anti-$A\beta_{1-17}$ (*lane 4, arrow*) while the peptide aggregation pattern resembled the one in *lane 3*. Densitometric evaluation of both complexes (*lanes 2* and *4, arrow*) indicated that the amount of apoJ-$A\beta_{1-40}$ formed was 4.6 times lower when aggregated peptide was used. To confirm this apparent different affinity of apoJ for aggregated and non-aggregated $A\beta_{1-40}$, inhibition assays were carried out on ELISA plates. Both fresh and aggregated peptides were allowed to interact with apoJ in fluid-phase for 3 h at 37 °C; the remaining free apoJ was tested for its ability to bind to $A\beta_{1-40}$-coated wells. As depicted in Fig. 4, aggregated $A\beta_{1-40}$ exhibited five times less efficiency to form complexes with apoJ (IC_{50} = 315 nM) than the fresh peptide (IC_{50} = 63 nM).

Competitive inhibition experiments using other plasma/CSF proteins with demonstrated binding affinity for Aβ (apoE2, apoE3, apoE4, ACT, Vn, and TTR) were performed by solid-phase assays. Increasing concentrations (0–2.5 μM) of each competitor were mixed with a constant amount of apoJ and immediately added to $A\beta_{1-40}$-coated wells. Bound apoJ was detected with monoclonal IF12 after 3 h of incubation. As indicated in Fig. 5, none of the proteins tested exhibited higher affinity for $A\beta_{1-40}$ than ApoJ. The competition curves distributed themselves into two very well defined groups: the first composed of the three apoE isoforms and the second contained the other proteins. ApoE2 (IC_{50} = 316 nM) was the strongest competitor of all the apoE isoforms according to the calculated IC_{50} values, followed by apoE3 (IC_{50} = 502 nM) and apoE4 (IC_{50} = 794 nM), indicating that they have 4–10 times lower relative affinity than apoJ for $A\beta_{1-40}$. None of the other proteins tested reached 50% inhibition of apoJ-$A\beta_{1-40}$ binding under the conditions tested (TTR, IC_{50} = 9550 nM; Vn, IC_{50} = 9820 nM; ACT, IC_{50} = 11340 nM).

TABLE I
Amino-terminal sequence analysis of the 85- and 170-kDa ApoJ-AB_{1-40} complexes

Yield is expressed in picomoles.

Step	Recovered		Theoretical		
	85 kDa	170 kDa	ApoJ (α)	ApoJ (β)	Aβ
	pmol				
1	Ser, 12.55 Asp, 30.47	Ser, 16.10 Asp, 37.80	Asp	Ser	Asp
2	Leu, 11.93 Gln, 13.26 Ala, 10.67	Leu, 13.96 Gln, 16.58 Ala, 13.66	Gln	Leu	Ala
3	Met, 7.43 Thr, 9.31 Glu, 9.28	Met, 9.27 Thr, 12.88 Glu, 11.71	Thr	Met	Glu
4	Pro, 13.51 Val, 11.93 Phe, 11.20	Pro, 11.07 Val, 10.48 Phe, 10.90	Val	Pro	Phe
5	Phe, 14.88 Ser, 9.30 Arg, ND[a]	Phe, 12.22 Ser, 7.99 Arg, ND	Ser	Phe	Arg
6	Ser, 9.82 Asp, 10.87 His, ND	Ser, 8.02 Asp, 9.44 His, ND	Asp	Ser	His
7	Pro, 5.17 Asn, 5.99 Asp, 6.22	Pro, 6.70 Asn, 4.19 Asp, 4.66	Asn	Pro	Asp
8	Tyr, 4.96 Glu, 4.99 Ser, 3.98	Tyr, 2.51 Glu, 3.26 Ser, 2.70	Glu	Tyr	Ser
9	Glu, 5.14 Leu, 4.92 Gly, 4.88	ND ND ND	Leu	Glu	Gly
10	Pro, 2.44 Glu, 2.46 Tyr, 2.21	ND ND ND	Glu	Pro	Tyr

[a] ND, not determined.

DISCUSSION

ApoJ (also known as clusterin or SP-40, 40) is a multifunctional disulfide-linked heterodimeric glycoprotein composed of two ~40-kDa subunits (named α and β chains) (28–30). The gene for apoJ maps to chromosome 8 (31–33). A single mRNA molecule (34) codifies a 449 amino acid chain, and the final apoJ structure is generated by post-translational cleavage at peptide bond Arg^{205}-Ser^{206}. ApoJ message is expressed in almost all mammalian tissues (35, 36), and the protein has been

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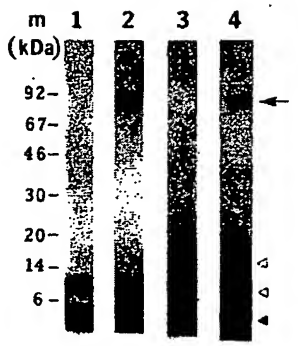

FIG. 3. **Immunodetection of the apoJ·Aβ$_{1-40}$ complex formed with fresh or aggregated peptide.** ApoJ·Aβ$_{1-40}$ and apoJ·Aβ$_{1-40}$(agg) complexes were prepared in PBS using 1.1 μmol of apoJ and 1.1 μmol of either fresh or 24-h heat-aggregated peptide. After 18 h incubation at 37 °C, complexes were separated on Tris-Tricine 10% SDS-PAGE and immunoblotted with anti-Aβ$_{1-17}$ (6E10). Visualization was carried out with peroxidase-labeled anti-mouse followed by ECL. *Lane 1*, fresh Aβ$_{1-40}$ (100 ng); *lane 2*, apoJ-fresh Aβ$_{1-40}$ complex (1:1 molar ratio); *lane 3*, 24-h heat-aggregated Aβ$_{1-40}$ (100 ng); *lane 4*, apoJ·Aβ$_{1-40}$(agg) complex (1:1 molar ratio). *Solid arrowhead*, monomeric Aβ$_{1-40}$; *open arrowheads*, dimeric and tetrameric forms of Aβ$_{1-40}$; *arrow*, apoJ·Aβ$_{1-40}$ complex.

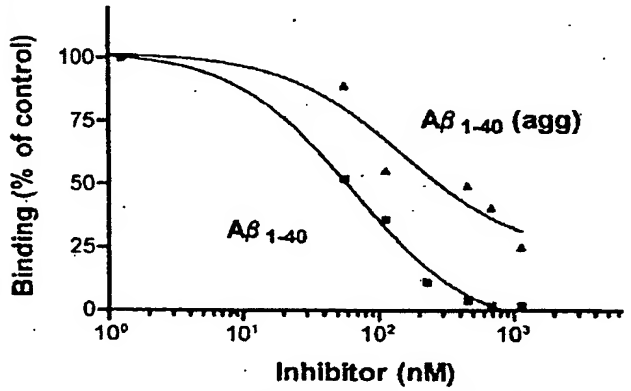

FIG. 4. **Inhibition of apoJ·Aβ$_{1-40}$ interaction by either fresh or aggregated Aβ$_{1-40}$.** ApoJ (25 nM) was preincubated with various concentrations of either fresh or 24-h-preaggregated Aβ$_{1-40}$ prior to the addition to Aβ$_{1-40}$-coated plates. Bound apoJ was detected as described under "Materials and Methods." ApoJ binding is expressed as percentage of binding compared to control wells incubated in the absence of inhibitor. Data represent the mean of three independent experiments duplicate experiments. S.D. never exceeded ±6%.

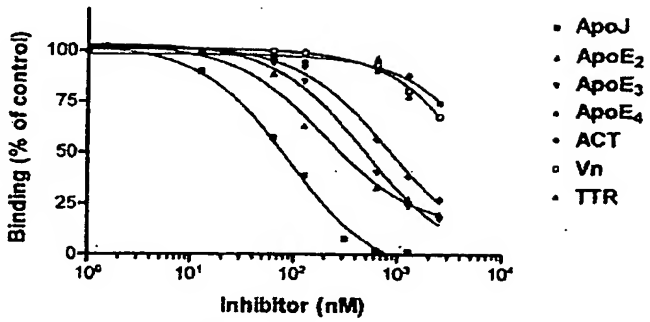

FIG. 5. **Competitive inhibition of the apoJ·Aβ$_{1-40}$ complex formation by amyloid associated proteins.** ApoJ (25 nM) was combined with variable concentrations (0–2500 nM) of apoE2 (▲), apoE3 (▼), apoE4 (◆), TTR (△), ACT (●), and Vn (□) and coincubated with Aβ$_{1-40}$-coated wells. Bound apoJ was determined as described under "Materials and Methods." The inhibition of biotin-labeled apoJ·Aβ$_{1-40}$ complex formation by native apoJ (■) presented on Fig. 1 (*inset*) is shown here for comparison. Results are expressed as percent of the maximum specific binding obtained in the absence of competitors. Data represent the mean of five independent duplicate experiments. S.D. never exceeded ±7%.

found in nearly all body fluids (36, 37). The normal concentration of apoJ in plasma ranges between 35 and 105 μg/ml (0.44–1.3 μM) (28), and it is primarily distributed in the high density lipoproteins (29); it is several times concentrated in seminal fluid while in CSF the values vary between 1.2 and 3.6 μg/ml (15–45 nM) (10, 38). ApoJ is involved in a variety of physiological processes, including lipid transport (36), secretion (39), membrane recycling (40), spermatogenesis (34, 35), and modulation of the complement activity (41). Within the central nervous system, apoJ is synthesized in neurons (42) and astrocytes (43, 44) and is able to cross the blood-brain barrier via a specific receptor/transport mechanism (45). Its production is up-regulated in the degenerative (46, 47) and/or regenerative processes (48, 49). The CSF concentration of apoJ is slightly elevated in AD patients (1.8–4.4 μg/ml), although the differences with the normal population are not statistically significant (10).

Biochemical data obtained *in vitro* indicate that apoJ is a major ligand for sAβ in plasma and CSF and that the complex apoJ-sAβ exists *in vivo* (18), suggesting that apoJ may act as a carrier protein for sAβ in plasma and CSF. Our results demonstrate that apoJ binds Aβ$_{1-40}$ with high affinity; the calculated K_d obtained from the saturation curve is 2×10^{-9} M. Monomeric and dimeric forms of apoJ (28) formed complexes with Aβ$_{1-40}$; both complexes were constituted by equimolar amounts of apoJ and Aβ$_{1-40}$, compatible with a 1:1 stoichiometry. The binding was specific and reversible; both, native apoJ and freshly prepared Aβ$_{1-40}$, inhibited the interaction of apoJ to immobilized Aβ$_{1-40}$ with a similar IC$_{50}$. The fact that 100% inhibition can be achieved by incubation of apoJ with Aβ$_{1-40}$ in solution indicates that the interaction indeed occurs in fluid-phase.

The self-aggregation rate of Aβ$_{1-40}$ is pH-, ionic strength-, temperature-, and concentration-dependent (50–52). The time course of aggregation of Aβ$_{1-40}$ in PBS at 37 °C, evaluated by SDS-PAGE, indicated that our freshly prepared peptide was mainly monomeric while the number of dimers, tetramers, and higher association forms increased as a function of incubation time, reaching a plateau at 24 h that remained without major changes for at least 72 h. ApoJ exhibited five times lower affinity for aggregated Aβ$_{1-40}$ than for freshly prepared peptide when tested by solid-phase ELISA and by gel electrophoresis followed by scanning evaluation. The presence of dimers, tetramers, and high molecular mass components indicated that apoJ was unable to reverse the aggregation of Aβ$_{1-40}$. All these data suggest that apoJ has the capability to bind with high affinity to non-aggregated forms of Aβ$_{1-40}$ but not to Aβ$_{1-40}$ polymers.

In order to balance the biological importance of the apoJ-sAβ interaction, competition experiments using other plasma/CSF proteins with demonstrated affinity to Aβ were carried out at physiologic pH. ApoE isoforms were 4–10 times less efficient than apoJ itself in inhibiting the formation of the complex apoJ·Aβ$_{1-40}$, being apoE4 the least avid competitor under the conditions tested. The rest of the proteins assayed (TTR, ACT, and Vn) exhibited an almost negligible competitive effect; none of them induced more than 10% inhibition at concentrations where apoJ in fluid-phase inhibited 100% the complex formation. It should be noted that all the proteins used in these competition experiments were either purified from plasma or

expressed in Sf9 cells. It would be interesting to determine whether other factors such as the association with other proteins, lipoproteins, and/or lipids may affect their interaction with Aβ. These data in the context of the mean physiologic concentrations of apoJ and apoE in plasma as well as in CSF ($apoJ_{plasma}$ = 0.87 ± 0.43 μM; $apoJ_{CSF}$ = 30 ± 15 nM; $apoE_{plasma}$ = 0.77 ± 0.26 μM; $apoE_{CSF}$ = 41 ± 16 nM) (10, 28, 53), suggest that normal physiologic conditions favor the formation of apoJ·Aβ complex. In fact, the presence of sAβ in apoJ-containing HDL particles was recently shown (54).

The data indicate the existence of a high affinity binding between apoJ and a peptide with identical primary structure to sAβ. It is conceivable that the interaction is not only related to the transport of the soluble peptide in plasma by apoJ-containing HDL but to the delivery of sAβ through the blood-brain barrier. In this regard, recent *in vivo* studies performed in guinea pigs have demonstrated the existence of cerebrovascular permeability for $Aβ_{1-40}$, human apoJ as well as for the apoJ·$Aβ_{1-40}$ complex (45, 55). Since apoJ has lower affinity for aggregated Aβ, the biological implications for the alteration of the interaction apoJ-sAβ under pathologic conditions should be further investigated as one of the possible mechanisms of peptide aggregation and deposition in AD tissue.

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THE SOLUBLE FORM OF ALZHEIMER'S AMYLOID BETA PROTEIN IS COMPLEXED TO HIGH DENSITY LIPOPROTEIN 3 AND VERY HIGH DENSITY LIPOPROTEIN IN NORMAL HUMAN PLASMA

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The amyloid fibrils of Alzheimer's neuritic plaques and cerebral blood vessels are mainly composed of aggregated forms of a 39 to 44 amino acids peptide, named amyloid beta (Aβ). A similar although soluble form of Aβ (sAβ) has been identified in plasma, cerebrospinal fluid and cell culture supernatants, indicating that it is produced under physiologic conditions. We report here that sAβ in normal human plasma is associated with lipoprotein particles, in particular to the HDL_3 and VHDL fractions where it is complexed to ApoJ and, to a lesser extent, to ApoAI. This was assessed by immunoprecipitation experiments of purified plasma lipoproteins and lipoprotein-depleted plasma and confirmed by means of amino acid sequence analysis. Moreover, biotinylated synthetic peptide $Aβ_{1-40}$ was traced in normal human plasma in *in vitro* experiments. As in the case of sAβ, biotinylated $Aβ_{1-40}$ was specifically recovered in the HDL_3 and VHDL fractions. This data together with the previous demonstration that $Aβ_{1-40}$ is taken up into the brain via a specific mechanism and possibly as an $Aβ_{1-40}$-ApoJ complex indicate a role for HDL_3- and VHDL-containing ApoJ in the transport of the peptide in circulation and suggest their involvement in the delivery of sAβ across the blood-brain barrier.

Amyloid beta (Aβ) is the major constituent of the fibrils composing senile plaques and vascular amyloid deposits in Alzheimer's disease (AD), Down's syndrome, congophilic angiopathy and Hereditary Cerebral Hemorrhage with Amyloidosis (Dutch type). It is a product (39-44 residues) generated from a larger precursor (amyloid precursor protein, βPP) through still unknown proteolytic pathways (for a review, see 1). Recently, soluble Aβ-like peptides (sAβ) were identified in cell culture supernatant of different cell lines, as

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well as in plasma and cerebrospinal fluid (CSF) obtained from normal and AD patients (2-4). Different physical and structural characteristics of Aβ have been clarified with the aid of synthetic peptides homologous to different regions of the molecule. In this sense, it has been demonstrated that peptides $A\beta_{1-40}$ and $A\beta_{1-42}$ and their shorter derivatives, $A\beta_{1-28}$ and $A\beta_{17-28}$ spontaneously form fibrils *in vitro*. Although peptide aggregation and fibril formation are greatly influenced by peptide concentration, pH, oxidation, ionic strength and temperature, as well as by the presence of amino acid substitutions it is still not clear why sAβ does not aggregate in biological fluids (5-11). It has been proposed that under normal conditions, the concentration of sAβ is too low to allow polymerization. Alternatively, it can be hypothesized that specific factors may maintain Aβ in solution impairing fibril formation. The latter is supported by the fact that spontaneous $A\beta_{1-40}$ fibril formation *in vitro* is inhibited in the presence of CSF (12).

We have described by means of direct binding experiments that synthetic peptide $A\beta_{1-40}$ immobilized on affinity matrices is able to retrieve apolipoproteins J (ApoJ) and E (ApoE) from plasma and CSF (13,14). The interaction of sAβ with these two apolipoproteins occurs *in vivo*, as demonstrated by the ability of anti-ApoJ (13) and anti-ApoE (unpublished observation) antibodies to co-precipitate sAβ from normal CSF in immunoprecipitation experiments. In addition, the blood-brain barrier regulates the transport of $A\beta_{1-40}$, ApoJ and ApoE; $A\beta_{1-40}$ appears to be taken up into the brain via a specific transport mechanism, possibly as a complex with ApoJ (15,16). Since ApoJ is primarily distributed in the high density lipoproteins in human plasma (17), we have tested the hypothesis that sAβ is transported complexed to these lipoprotein particles in the circulation. Direct immunoprecipitation of endogenous sAβ and biotinylated-$A\beta_{1-40}$ (used as a tracer) from different lipoprotein (LP) fractions as well as from lipoprotein-depleted plasma (LPDP) indicate that in normal human plasma sAβ is complexed to the HDL_3 and VHDL , mainly in association with ApoJ.

MATERIALS AND METHODS

Synthetic peptides: Peptide DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVV ($A\beta_{1-40}$ positions 672-711 of βPP_{770}) was synthesized at the W. M. Keck Facility at Yale University using solid-phase tBOC (N-tertbutyloxycarbonyl)-chemistry. Amino acids were coupled by activated esters and final deprotection/cleavage was performed using hydrogen fluoride. Crude peptide was purified via HPLC using a Vydac C18 reverse phase column and a linear gradient of 20-80% acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 1.0 ml/min. The final preparation of the peptide was lyophilized and characterized by analytical reverse-phase HPLC, amino acid analysis and laser desorption mass spectrometry. $A\beta_{1-40}$ was biotinylated with Sulfo-NHS-Biotin (Pierce; Rockford, Il) according to the manufacturer's specifications. Briefly, one milligram of $A\beta_{1-40}$ was

dissolved in 1 ml of 50 mM Sodium Bicarbonate Buffer, pH 8.5, containing 30% acetonitrile and allowed to react with 0.32 mg of Sulfo-NHS-Biotin for 2 h at 4°C. Unreacted Biotin was removed from the mixture by centrifugation at 1000 x g for 30 min using Centricon 3 Microconcentrator (Amicon; Beverly, MA). The sample was diluted in 20 mM Tris-HCl pH 7.4, containing 150 mM NaCl (TBS) and 30% acetonitrile, aliquoted and stored at -70°C.

Antibodies: Polyclonal anti-SP28 (anti-$A\beta_{1-28}$) was available in the laboratory (18). Polyclonal SGY2134 (anti-$A\beta_{1-40}$) was a kind gift from S. Younkin (2). Monoclonal anti-ApoE antibody was obtained from A. Dergunov (19) and monoclonal 6E10 (anti-$A\beta_{1-17}$) was obtained from K. S. Kim (20). Monoclonal antibody IF12 (anti-ApoJ, α-chain) was kindly provided by Nam-Ho Choi-Miura (21). Monoclonal antibodies anti-ApoAI (type 3) and anti-ApoB as well as polyclonal antibodies anti-serum amyloid A (SAA), anti-ApoE and anti-α1-antichymotrypsin (ACT) were from Chemicon Int. (Temecula, CA).

Lipoproteins isolation: Human LPs were isolated by means of preparative gradient ultracentrifugation (22) either from human plasma obtained from normal healthy subjects, ages 25 to 40 years after 10-12 h fast or from plasma obtained from normal donors at the University Hospital Blood Bank. In all cases, centrifugations were performed at 16°C at 40,000 rpm using a Beckman Ti 50.2 rotor and densities were adjusted to the necessary values with solid KBr. Briefly, starting plasma was first subjected to ultracentrifugation for 16 h at its original δ=1,0063 g/ml in order to obtain the VLDL fraction. LDL were then floated over the same period of time at δ=1.063 g/ml. Infranatants were re-adjusted to δ=1,125 g/ml and centrifuged for 20 h. The upper phase of HDL_2 was removed, the density increased to 1,21 g/ml and HDL_3 were recovered after 40 h centrifugation. The final adjustment to δ=1,25 g/ml rendered VHDL floating after 40 h of centrifugation. The infranatant of the δ=1.25 g/ml, named lipoprotein-depleted plasma or LPDP, was also collected and used in the experiments. All the fractions were extensively dialyzed against TBS buffer containing 1mM EDTA and stored at 4°C until used. The protein content of the LP and LPDP preparations was measured using the Bradford method (BioRad; Richmond, CA) employing bovine serum albumin as a standard. Each fraction was characterized by Tris-Tricine/12,5%-polyacrylamide gel electrophoresis followed by immunoblotting with specific antibodies directed against different apolipoproteins. In a separated experiment, 2 μg of biotinylated $A\beta_{1-40}$ were added to 20 ml of human plasma and the mixture was incubated for 3 h at 37°C followed by LP isolation by preparative gradient ultracentrifugation, in identical conditions as described above.

Immunoprecipitation: Forty milligrams of each LP fraction (2 mg in the case of LP-containing biotin-labeled $A\beta_{1-40}$) were mixed (1:5 vol/vol) with 5x RIPA buffer (50 mM Tris-HCl pH 8.0 containing 150 mM NaCl / 1% Triton X-100 / 0.5% cholic acid / 0.1% SDS / 5 mM EDTA / 1mM phenylmethane-sulphonyl fluoride / 1 μg/ml leupeptin / 0.1 μg/ml pepstatin / 1 μg/ml Nα-p-tosyl-L-lysine chloromethyl ketone) and all the fractions were then adjusted to a final volume of 14 ml with 1x RIPA (when necessary, non-denaturing immunoprecipitation was carried out in TBS). One of the following antibodies in dilution 1:500 (SGY2134, anti-SP28, anti-ACT, anti-SAA, anti-ApoAIV, IF12, anti-ApoE or anti-ApoAI) was added together with 50 μl of Protein G-Agarose beads equilibrated in RIPA buffer and incubated at 4°C for 12 h on a rocking platform. After washing the beads three times with TBS, the immunoprecipitated proteins were separated by Tris-tricine / 12.5% SDS-PAGE and transblotted to 0.45 μm nitrocellulose membranes (BioRad) for 2 h at 400 mA using 3-cyclohexylamino-1-propanesulphonic acid pH 11, containing 10% methanol. Membranes were blocked with 6% bovine serum albumin and labelled with monoclonal 6E10 (1:300) overnight at 4°C. Anti-mouse Peroxidase-labeled (Amersham, 1:3000) was used as a secondary antibody. Fluorograms were prepared with the Western Blot Chemiluminescence reagent (DuPont; Wilmington, DE) according to the manufacturer's specifications. For control purposes, 50 ng of $A\beta_{1-40}$ in a volume of 14 ml were immunoprecipitated with anti-SP28. In the case of biotinylated-$sA\beta_{1-40}$, Peroxidase-

labeled Streptavidin (Amersham, 1:700) followed by ECL was used for detection purposes. Before immunoprecipitation of the LPDP fraction, native IgG was removed by incubation with Protein G-Sepharose for 3 h at 4°C. For amino acid sequence analysis, proteins were separated on Tris-tricine SDS-PAGE and transferred onto Immobilon P membranes (Millipore, Bedford, MA) in identical conditions as described above. After staining with Coomassie blue, bands were excised and sequenced on a 477A protein sequencer (Applied Biosystems; Foster City, CA).

RESULTS AND DISCUSSION

Two apolipoproteins, ApoJ and ApoE have been found to interact with Aβ. Affinity matrices prepared with synthetic peptides homologous to Aβ demonstrated binding interaction to both apolipoproteins from either plasma or CSF (13,14,23). In addition, the existence of both complexes (ApoJ-sAβ and ApoE-sAβ) was demonstrated *in vivo*; immunoprecipitation with anti-ApoJ and anti-ApoE antibodies recovered sAβ from CSF. We hypothesized that such interactions may take place in ApoJ- or ApoE-containing LP species of human plasma. In order to test this hypothesis, we have purified VLDL, LDL, HDL_2, HDL_3 and VHDL from normal human plasma and assessed the purity of the various fractions by means of immunoblot analysis (Table I). Forty milligrams of each purified LP and LPDP fractions were subjected to immunoprecipitation with SGY2134 anti-Aβ antibodies and the resulting precipitates were tested for Aβ immunoreactivity with monoclonal 6E10 antibody. As indicated in Figure 1, sAβ was specifically immunoprecipitated from HDL_3 (lane 4) and VHDL (lane 5) fractions, while the VLDL (lane 2), LDL (lane 3) and LPDP (lane 6) fractions were unreactive. Immunoprecipitation carried out with the HDL_3 and VHDL fractions combined retrieved a stronger immunoreactivity signal at the same molecular weight (lane 7). The 4.5 kDa band detected by the 6E10

TABLE I : IMMUNOCHEMICAL CHARACTERIZATION OF PURIFIED LIPOPROTEIN FRACTIONS. An aliquot of each lipoprotein fraction (50 μg) was subjected to gel electrophoresis and immunoblotting analyzed with antibodies against ApoB, ApoE, ApoAI, ApoJ and SAA. The presence of each apolipoprotein in the different fractions is expressed in relation to their content in the other fractions.

Apolipoprotein	VLDL	LDL	HDL_2	HDL_3	VHDL
ApoB	+	++	–	–	–
ApoE	+++	+++	+	+	–
ApoAI	–	–	++	+++	++
ApoJ	–	–	–	++	+
SAA	–	–	+	++	–

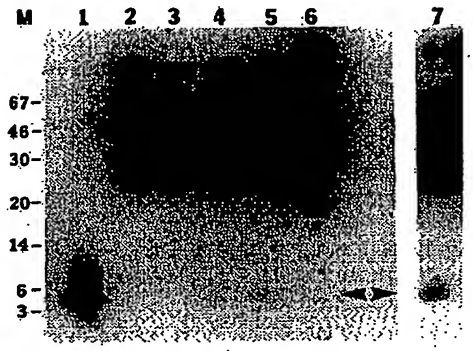

FIGURE 1. Immunoprecipitation of sAß from LP fractions of normal human plasma. Analysis was performed as in Materials and Methods. Soluble Aß (arrowhead) was specifically precipitated by SGY2134 polyclonal antibodies only from HDL3 (lane 4) and VHDL (lane 5) and not from VLDL (lane 2), LDL (lane 3),or LPDP (lane 6). Fractions HDL_3 and VHDL combined retrieved a stronger anti-Aβ immunoreactivity signal (lane7). One hundred nanograms of synthetic peptide $A\beta_{1-40}$ (lane 1) was used as a positive control. Molecular weight markers (in kDa) are shown on the left side of the picture.

antibody showed an identical electrophoretic mobility to the monomeric form of the synthetic peptide $A\beta_{1-40}$ (lane 1).

In order to confirm the association of sAβ with the HDL_3 and VHDL fractions, we have traced biotinylated $A\beta_{1-40}$ in human plasma using *in vitro* experiments. Twenty milliliters of plasma from normal healthy subjects were incubated with 2.0 μg of biotinylated $A\beta_{1-40}$; after purification of the different LP fractions, identification of the biotin-labeled peptide was performed by immunoblot analysis following immunoprecipitation with anti-SP28. As depicted in Figure 2, biotinylated $A\beta_{1-40}$ was specifically recovered in the HDL_3 (lane 5), VHDL (lane 6) and to a much lesser extent HDL_2 (lane 4) while it was absent in the LPDP, VLDL and LDL fractions (lanes 1 to 3, respectively). Immunoprecipitation experiments carried out with the combined HDL_3 and VHDL fractions employing a set of different antibodies (anti-ApoJ, anti-ApoAI, anti-ApoE, anti-SAA and anti-ACT) was aimed to determine whether biotinylated $A\beta_{1-40}$ was associated to any of the major components of the lipoprotein particles. As shown in Figure 3, anti-ApoJ (lane 2) and scarcely anti-apoAI (lane 3) retrieved the biotinylated peptide from the same HDL_3 and VHDL fractions, while all others antibodies gave negative results (lanes 4-6). The amount obtained by anti-ApoAJ was comparable with the amount recovered with anti-SP28 (lane 1) while the amount recovered by anti-ApoAI represents less than 1% of the total. Under these conditions, anti-ApoE was unable to co-precipitate biotinylated $A\beta_{1-40}$ although the existence of the complex has been demonstrated in CSF.

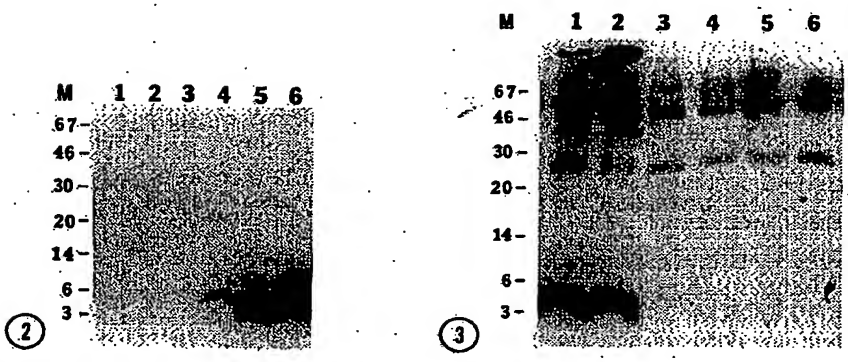

FIGURE 2. Immunoprecipitation of tracer biotin-labeled Aß1-40 from LP fractions of human plasma. Analysis was performed as described in Materials and Methods. Biotinylated $A\beta_{1-40}$ was specifically precipitated from the different LP fractions by polyclonal anti-S28. The biotin-labeled peptide was absent in LPDP (lane 1), VLDL (lane 2), LDL (lane 3), present in very low concentration in HDL_2 (lane 4) and strongly positive on HDL_3 (lane 5) and VHDL (lane 6). Molecular weight markers (in kDa) are shown on the left side of the picture.

FIGURE 3. Immunoprecipitation of tracer biotin-labeled Aß1-40 from HDL_3 + VHDL fractions of human plasma. Analysis was performed as described in Materials and Methods. Biotinylated peptide was precipitated from combined HDL_3 and VHDL fractions by polyclonal anti-Aß1-28 (lane 1) and monoclonal anti-ApoJ (lane 2) but not by monoclonal anti-ApoE (lane 4) and polyclonal anti-SAA (lane 5) and anti-ACT (lane 6) antibodies. Trace amounts of biotinylated peptide were precipitated with monoclonal anti-ApoAI (lane 3). Molecular weight markers (in kDa) are shown in the left side of the picture.

Immunoprecipitation experiments performed under non-denaturing conditions (TBS) with both anti-Aβ (SGY2134) and anti-ApoJ (IF12) antibodies recovered a similar protein pattern. In both cases, two components of 28 kDa and 38-40 kDa were detected under reducing conditions by Coomassie blue staining (not shown). The proteins were identified as ApoAI and ApoJ; amino acid sequence analysis obtained from the 28 kDa band yielded the sequence DEPPQSPWDRVK (ApoAI N-terminus) while the one acquired from the 38-40 kDa component yielded the sequences DQTVSDNELQ and SLMPFSPYEP (corresponding to the N-terminus of ApoJ α- and β-chains, respectively).

Apolipoprotein J in human plasma is associated with high density lipoproteins (17). The ApoJ-containing species are relatively poor in lipids: the protein content represents up to 78-89% while lipids account for 11-22% of the lipoprotein mass. Of the lipids, cholesterol and phospholipids are predominant; triglyceride represents only 1% of ApoJ-HDL lipids. ApoAI is the major protein found in HDL fractions and the ApoAI tightly associated with ApoJ represents only 2-4% of the total ApoAI in plasma. The association

appears to be of hydrophobic nature, requiring nonionic detergents for dissociation (17,24). Due to this association, it is possible that the bulk of the ApoAI immunoprecipitated from the HDL_3 + VHDL fractions by anti-Aβ antibodies under non-denaturing conditions was co-precipitated with ApoJ. However, the small amount of biotinylated $A\beta_{1-40}$ obtained by immunoprecipitation with anti-A antibody under denaturing conditions might reflect a protein - protein interaction between ApoAI and $A\beta_{1-40}$. Preliminary binding experiments of purified ApoAI to immobilized $A\beta_{1-40}$ indicate the existence of this binding activity, although the affinity values are much lower than for the interaction ApoJ-$A\beta_{1-40}$ (manuscript in preparation).

Different circulating proteins have been implicated in Aβ-binding activity, among them ACT (25), ApoJ (13), ApoE (14,23) and transthyretin (26), although their influence in amyloidogenesis is controversial. The data presented here indicate that sAβ in plasma of normal individuals is complexed to HDL_3 and VHDL, mainly in association with ApoJ. It is possible that the interaction of sAβ with high density lipoproteins is not only related to the transport of the peptide in plasma but to the delivery of sAβ through the blood-brain barrier. In this sense, recent *in vivo* studies performed in guinea pigs demonstrated the existence of cerebrovascular permeability for $A\beta_{1-40}$, human ApoJ as well as for ApoJ-$A\beta_{1-40}$ complex, while human ApoE seems to have very limited access across the blood-brain barrier (15,16). The significant blood-to-brain permeability to ApoJ and its complex with $A\beta_{1-40}$ suggest the presence of a specific receptor/transport mechanism, the protein gp330 (ApoJ receptor, ref. 27) being one of the likely candidates. The biological implications for the alteration of the interaction ApoJ-sAβ under pathologic conditions should be further investigated as one of the possible mechanisms of peptide aggregation and deposition in AD tissue.

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Detection of Apolipoprotein E/Dimeric Soluble Amyloid β Complexes in Alzheimer's Disease Brain Supernatants

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The inheritance of the apolipoprotein (apo) E4 allele is an important risk factor for late-onset Alzheimer's disease (AD). A major component of the Alzheimer's disease neuritic plaques is amyloid β (Aβ). We previously identified apoE/Aβ complexes within neuritic plaques (1). It was not known if this interaction takes place before or after Aβ peptides become incorporated into neuritic plaques. To address this question we sought evidence of apoE complexes with brain soluble Aβ peptides in AD and control patients. In addition, numerous proteins have been shown to bind Aβ peptides *in vitro*. It is not know if any of these bind brain sAβ *in vivo*. We found evidence for the presence of apoE/dimeric sAβ complexes in the AD brain and could not detect complexes with other Aβ peptide binding proteins. The binding of sAβ to apoE may be one factor influencing its clearance from the brain and/or its conformational state. © 1997 Academic Press

The defining neuropathological lesions of AD are the deposition of amyloid β (Aβ) in the form of amyloid fibrils in congophilic angiopathy and senile plaques, as well as the aggregation of abnormally phosphorylated tau into neurofibrillary tangles (2,3). The Aβ contained in senile plaques is characterized by an altered conformational state that is predominantly β pleated sheet. Aβ peptides can also exist as a normal soluble protein (sAβ) in biological fluids (2). The major sequence of sAβ is 1-40 but longer and shorter Aβ peptides are also found (4). Synthetic peptides homologous to Aβ1-40 have been shown to be able to cross the blood-brain-barrier (BBB) both from the systemic circulation into the brain and from the brain out systemically (5-7). Clearly one factor that can affect the deposition of Aβ peptides as amyloid, in the extracellular space of the brain, is a reduced clearance across the BBB or alterations of Aβ peptide uptake by cell surface receptors. Aβ peptide binding proteins are likely to affect both these mechanisms of brain Aβ peptide clearance. In the CSF and plasma of normal individuals the major sAβ binding protein appears to be apolipoprotein J (8-10). However, in the brain, it is not known what are the sAβ binding proteins. One Aβ peptide binding protein, identified by in vitro studies, is apolipoprotein E (apoE). ApoE is a 34-kD product of a four exon gene located on the long arm of chromosome 19. In humans, the apoE gene is polymorphic, leading to 3 major apoE isoforms, namely E2, E3 and E4. ApoE3, the most common isoform, has a cysteine at position 112 and arginine at position 158; apoE2 is the least frequent isoform, with cysteine at both positions, whereas apoE4 possesses arginine at both sites (11). A strong association between the inheritance of the apoE allele E4 and both sporadic AD and late-onset FAD has been described in numerous publications (12,13). The *in vitro* studies showing Aβ peptides binding to apoE and the observation that the inheritance of the E4 allele correlates with increased deposition of Aß in AD patients, suggests a direct apoE/Aβ interaction (14,15). *In vivo*, we and another group have provided evidence that this apoE/Aβ interaction occurs within senile plaques (1,16). Both studies showed that apoE co-purifies with Aβ peptides during biochemical extractions of amyloid from neuritic plaques. Whether apoE bindings to Aβ before or after it is deposited in amyloid plaques is not known. In this study we sought to address this question, as well as the identification of possible brain sAβ binding proteins, which may influence brain sAβ clearance, in brain supernatants of AD and control patients.

MATERIALS AND METHODS

Brain tissue preparation. The cerebral cortex from 3 AD patients and 2 age-matched control subjects were analyzed. The AD patient's

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brains fulfilling the CERAD criteria of AD (17). The brain tissue was subject to apoE isotyping using our published protocol (18). In order to produce a brain sAβ containing fraction, the brain tissue was treated similar to prior descriptions (19,20). Grey matter was dissected free of vessels. The material was homogenized in a glass homogenizer in 4 volumes (wt/vol) of TBS buffer (Tris: 20 mM; NaCl: 150mM; EDTA 5mM) containing protease inhibitors (Complete, Boehringer-Mannheim) and subjected to ultracentrifugation (100,000g, 60 mins). Resulting supernatants were collected, dialyzed against milliQ water over 48 hours and then lyophilized. Lyophilized material corresponding to a volume of 5 ml of brain supernatant was resuspended in an equal volume of PBS buffer (10 mM phosphate, 150 mM NaCl) containing protease inhibitor.

Immunoprecipitation of sAβ and apo E. For immunoprecipitation of Aβ, 50 μl of paramagnetic dynabeads M-450 coated with goat anti-mouse IgG (Dynal) were incubated over 3 hours at room temperature with 5 μl of 4G8 (Senetek) and 5 μl of 6E10 (21). 4G8 and 6E10 are monoclonal antibodies recognizing Aβ residues 17-24 and 1-17, respectively (21). For immunoprecipitation of apo E, 50 μl of magnetic beads were first incubated with 10 μl of monoclonal anti-apoE 3D12 (Biodesign Int.). The magnetic beads were then washed three times with PBS buffer containing 0.1% of bovine serum albumin. Brain supernatants (5 ml) were incubated in presence of saturated beads overnight at 4°C. After three PBS washes, the beads were either resuspended in Laemmli sample buffer for SDS-PAGE under reducing conditions, or dried on a speed vac for mass spectrometry analysis. Pooled cerebrospinal fluid (CSF) from non-AD control subjects, synthetic Aβ1-40 in PBS at 50ng/ml and PBS alone were used as controls for the immunoprecipitations of brain supernatants. Controls also included brain supernatants, immunoprecipitated with dynabeads alone. These controls were subject to SDS-PAGE and mass spectroscopy. A variety of other antibodies was also used for immunoprecipitation including: monoclonal anti-apoJ (8), monoclonal anti-α_1-antichymotrypsin (ACT; 8E6, Biodesign Int.), polyclonal anti-transthyretin (TTR; Atlantic antibodies) and anti-serum amyloid P component (SAP; Sigma).

Immunoblot analysis. Samples were separated on a 16% tris-tricine SDS-PAGE and electroblotted onto PVDF membrane (Amersham) over 1 hour at 400 mA constant current using 3-cyclohexylamino-1-propanesulphonic acid, pH 11, containing 10% methanol. Membranes were blocked in PBS containing 0.1% Tween 20, 5% non-fat milk and incubated overnight in 4G8 or 6E10 at 1:500 in PBS, 1% BSA, 0.1%Tween-20, followed by anti-mouse, horseradish peroxidase conjugated, secondary antibody at 1:2000 (Amersham). Blots were visualized using enhanced chemiluminescence (ECL, Amersham). For immunoblot analysis, we also used goat polyclonal anti-apoE (Chemicon) at 1:500 dilution followed by incubation in presence of horseradish peroxidase conjugated, anti-goat IgG (Vector) at 1:2000 dilution.

Mass spectrometry analysis. Immunoprecipitated samples were also subjected to laser desorption mass spectroscopy (LDMS) on a custom built machine at the Skirball Institute at NYU Medical Center (22). Beads were washed three times in PBS, followed by speed-vac drying. The immunoprecipitated material was then eluted by 10 μl of water:isopropyl alcohol:formic acid (4:4:1). Five μl were subjected to LDMS using α-cyanno-4-hydroxycinnamic acid as a matrix (23).

RESULTS

Apolipoprotein E Isotyping

The three AD brains had an apoE 3/3 isotype, while the two age-matched controls were 3/3 and 3/2.

Presence of sAβ Peptide in AD Brain Supernatants

Analysis of brain sAβ in cerebral cortex was performed by Western blotting and LDMS. Western blotting was done using 6E10 for Aβ detection (fig 1A). A 4 and 8 kDa band immunoreactive with the anti-Aβ 6E10 were evident in the immunoprecipitant from AD brain supernatant (figure 1A, lane 1). The immunoprecipitant from CSF showed only a prominent 4kDa Aβ immunoreactive band (figure 1A, lane 2). Controls included immunoprecipitations of synthetic Aβ1-40 at 50ng/ml in PBS, which also showed the same 4 and 8kDa immunoreactive bands (figure 1A, lane 3). Synthetic Aβ1-40 (20ng) freshly dissolved in sample buffer, also showed the same 4 and 8 kDa Aβ immunoreactive bands (figure 1A, lane 4). Immunoprecipitated samples were also analyzed by LDMS (figure 1B). Aβ1-40 is the major species present in AD brain supernatant (figure 1B, I), giving a peak of of 4329.3 (expected m/z of Aβ1-40 is 4329.9). In CSF (figure 1B-II) Aβ1-40 (observed m/z 4330.2; expected m/z 4329.9), 1-38 (observed m/z 4131.6, expected m/z 4131.6) and 1-42 (observed m/z 4515.3, expected m/z 4514.1) were detected. Synthetic Aβ 1-40 showed a mass of 4329.5 (figure 1B-III). The intensity of the sAβ peaks obtained in CSF were consistently higher than that obtained from brain supernatants, even thought the concentration of brain sAβ by ELISA was higher in the brain supernatants (data not shown). This was also true for equal quantities of synthetic Aβ1-40 that were added to CSF versus brain supernatants. Hence the sensitivity of our method is lower in brain supernatants, explaining why we detect only sAβ1-40 in brain supernatants, while in CSF several sAβ peptide species are evident.

Immunoprecipitation of Control, Age-Matched Brain Supernatants

Analysis of control brain supernatants was done using the same procedures as for the AD brains. Immunoprecipitation using anti-Aβ antibodies 4G8 and 6E10 failed to show an Aβ immunoreactive band on Western blotting (figure 2A, lane1), and no Aβ peptides peaks were noted by LDMS (data not shown). As expected the anti-apoE antibodies, immunoprecipitated apoE (figure 2B, lane2, see 34kDa band); however, these antibodies did not co-immunoprecipitate Aβ peptides (figure 2A, lane2). These results indicate that the levels of brain sAβ and sAβ/apoE complexes are too low to be detected in normal brain supernatants.

ApoE/Aβ Complexes in AD Brain Supernatants

Using the anti-apo E (3D12, Biodesign Int.), we were able to immunoprecipitate apo E from AD brain supernatants (figure 3A, lane 1). Significantly apo E could also be immunoprecipitated by anti-Aβ anti-

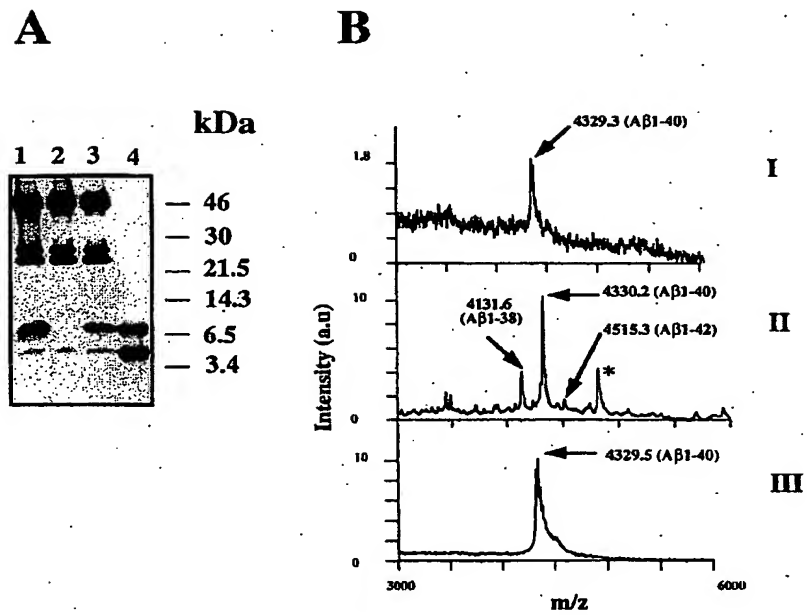

FIG. 1. Western blot (A) and mass spectrometry analysis (B) of sAβ present in AD brain supernatants and in CSF. (A): AD brain supernatants were immunoprecipitated using monoclonal anti-Aβ antibodies: 4G8/6E10 (21) and goat-anti-mouse magnetic beads (Dynal). 5 ml of pooled, non-AD CSF and 5 ml of phosphate buffered saline (PBS), containing synthetic A$\beta_{1\text{-}40}$ (50 ng/ml) were processed the same way and used as controls. The immunoprecipitants were separated on a 16 % tris-tricine gel electrophoresis, transferred onto PVDF membrane and immunoblotted with anti-Aβ antibody 6E10 (21). Visualization was carried out by ECL (Amersham) . In lane 1 AD brain supernatant immunoprecipitant was loaded. A 4 and 8 kDa band corresponding to monomeric and dimeric Aβ are evident. The 8kDa band is more prominent. Lanes 2 and 3 were loaded with immunoprecipitant from CSF and PBS containing A$\beta_{1\text{-}40}$ (50 ng/ml) respectively. A 4 kDa band is seen in lane 2 corresponding to monomeric Aβ. In lane 4, 20 ng of synthetic Aβ1-40 was run and 4 and 8kDa bands are seen. The heavy higher molecular weight bands seen at 24, 28 and 50 kDa correspond to the heavy and light chains of immunoglobulin. (B) Analysis by LDMS of the immunoprecipitated material. I) AD brain supernatant. II) pooled non-AD CSF. III) Synthetic Aβ1-40. Aβ1-40 is the major species present in AD brain supernatant (fig 1B, I), giving a peak of of 4329.3 (expected m/z of Aβ1-40 is 4329.9). In CSF (fig 1B-II) Aβ1-40 (observed m/z 4330.2; expected m/z 4329.9), Aβ1-38 (observed m/z 4131.6, expected m/z 4131.6) and Aβ1-42 (observed m/z 4515.3, expected m/z 4514.1) were detected. An additional peak is noted (indicated by an asterix), that does not match to an Aβ peptide. This peak was also seen in some controls when PBS was immunoprecipitated with beads alone; hence this peak corresponds to a contaminant in the magnetic beads. Synthetic Aβ 1-40 showed a mass of 4329.5 (fig 1B-III).

bodies 4G8 and 6E10 (figure 3A, lane 2). Conversely, dimeric sAβ was immunoprecipitated by anti-apoE antibody 3D12 (Biodesign Int.) (figure 3B, lane 2). AD brain supernatants were also immunoprecipitated with 4G8/6E10 (figure 3B, lane 1). In both samples, Western blot analysis using 4G8 shows one band located at 8 kDa corresponding to the dimer form of Aβ. The identity of this 8kDa Aβ immunoreactive band was confirmed by LDMS. LDMS showed a peak of 4327.9, corresponding to Aβ1-40 (expected m/z 4329.9) (figure 3C). LDMS shows only the monomeric form of Aβ1-40, since the solution used to elute the material from the magnetic beads (water:isopropyl alcohol:formic acid, 4:4:1) disrupts the dimers. This is also true of aggregated synthetic Aβ1-40. Using the same experimental procedures anti-apoJ, anti-SAP, anti-ACT and anti-TTR antibodies failed to immunoprecipitate sAβ, evaluated both by Western blotting and LDMS (data not shown).

DISCUSSION

Our results clearly show that brain sAβ is complexed, in part to apoE. This was established in our immunoprecipitation experiments by showing that anti-apoE antibodies can co-precipitate sAβ and conversely monoclonal anti-Aβ antibodies can immunoprecipitate apoE in brain supernatants. The identity of the immunoprecipitating sAβ was determined by Western blotting and by mass spectroscopy. Under the same conditions, we did not find evidence of brain sAβ complexes with other proteins such as apo J, serum amyloid P component, α_1-antichymotrypsin and transthyretin; each of which is known to bind Aβ peptides *in vitro*. In control brain supernatants we failed to found such complexes, suggesting that either in these brains such complexes do not exist or that our methods are not sensitive enough to detect lower levels of these complexes in normal brains. Previously we have established that Aβ extracted from

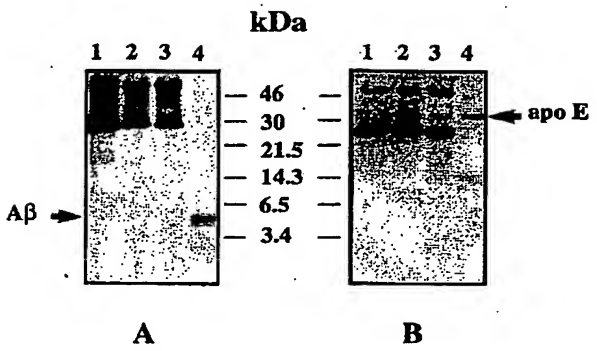

FIG. 2. Shows Western blots of immunoprecipitations using control, age-matched brain supernatants. In A, the primary antibody used was monoclonal, anti-Aβ antibody 4G8, while in B the same blot was developed using polyclonal anti-apoE (Chemicon). In lane 1 (A and B) immunoprecipitants using monoclonal anti-Aβ 4G8/6E10, was loaded . Lane 2 and 3 (A and B) were loaded with immunoprecipitants using anti-apoE (3D12, Biodesign Int.) and anti-ACT (8E6, Biodesign Int.) respectively. In A and B lane 4, 10 ng of synthetic Aβ1-40 and 20ng of recombinant apoE (PanVera) were loaded respectively. In A, a 4 kDa band corresponding to the synthetic Aβ1-40 is seen in lane 4 only. No Aβ immunoreactive band was seen in any of the control brain supernatant immunoprecipitation experiments. In B, a 34kDa apoE immunoreactive band is seen in lanes 2 and 4, corresponding to the control brain supernatant immunoprecipitated apoE and the recombinant apoE respectively. The heavy higher molecular weight bands seen at 24, 28 and 50 kDa correspond to the heavy and light chains of immunoglobulin.

neuritic plaques, is in part complexed to apoE (1). The presence of apoE in neuritic plaques may only be related to the know "sticky" nature of amyloid deposits, with the apoE becoming associated with the Aβ only after it is deposited. However, our results would suggest that apoE can bind dimeric sAβ peptides prior to their parenchymal deposition.

The binding of apoE to brain sAβ may be one important determinant of the sAβ clearance from the brain and/or from the brain's extracellular space, where amyloid deposition occurs. Interestingly it has been shown in a guinea-pig model that Aβ1-40 peptides complexed to apoE has a greatly reduced transport across the blood-brain barrier (BBB), compared with Aβ peptide alone or Aβ peptides complexed to apoJ (5,24). Hence, in the AD brain the formation of brain sAβ-apoE complexes may be one factor reducing clearance of sAβ peptides from the brain, across the BBB. The current study did not address any differences in the levels of brain sAβ-apoE complexes related to apoE isotypes; such studies are in progress. The apoE isotype of each of the three AD brains we examined was 3/3, while the two controls had a 3/3 and 3/2 isotype. However, differential binding of the three apoE isotypes to Aβ peptides may influence sAβ clearance from the brain. The binding of delipidated apoE3 and E4 to Aβ has the same K_D (25), while lipidated apoE3 in gel shift assays, appears to bind Aβ peptides better than lipidated apoE4 (26-29). The latter finding has been used to suggest that apoE3, with its better binding to Aβ, may clear sAβ more quickly from the brain, via interac-

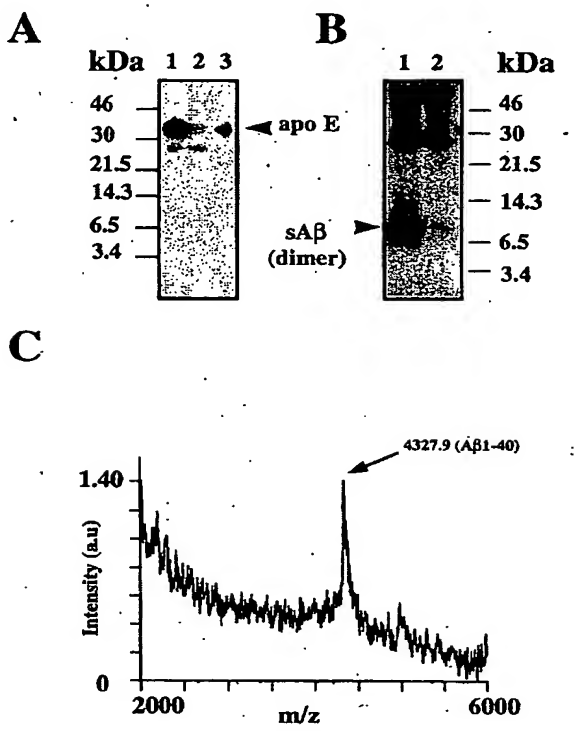

FIG. 3. Western blot (A, B) and mass spectrometry (C) analysis of the apo E/sAβ complex in AD brain supernatants. (A) AD brain supernatant was subject to immunoprecipitation with either monoclonal anti-apoE (3D12, Biodesign Int.) (lane 1) or monoclonal anti-Aβ 4G8/6E10 (lane 2). Immunoprecipitatants were separated on a 16% tris-tricine gel electrophoresis, transferred onto PVDF membrane and immunoblotted using a goat polyclonal anti-apo E (Chemicon). Lane 1 shows AD brain supernatant immunoprecipitated by anti-apoE. Lane 2 shows AD brain supernatant immunoprecipitated by anti-Aβ 4G8/6E10. In lane 3, 5 ng of apo E purified from plasma was loaded. A 34kDa band corresponding to apoE is seen in lanes 1-3. Significantly the monoclonal anti-Aβ antibodies also immunoprecipitated apoE (see lane 2). The 25 and 50 kDa bands seen in lanes 1 and 2 correspond to the heavy and light chains of immunoglobulin; these bands are less intense than seen in figures 1, 2 and 3B, since this PVDF membrane was exposed for a shorter time. (B) AD brain supernatant was subject to immunoprecipitation with either monoclonal anti-Aβ 4G8/6E10 (21) (lane 1) or monoclonal anti-anti-apoE (3D12, Biodesign Int.) (lane 2). Immunoprecipitatants were separated on a 16% tris-tricine gel electrophoresis, transferred onto PVDF membrane and immunoblotted using monoclonal anti-Aβ 4G8 (21). An 8 kDa Aβ immunoreactive band is evident in lanes 1 and 2. The 25 and 50 kDa bands seen in lanes 1 and 2 correspond to the heavy and light chains of immunoglobulin. (C) Shows the LDMS peak obtained for the immunoprecipitant from AD brain supernatant using monoclonal anti-apoE antibodies. This represents the same material which was run in lane 2 of the Western blot B. The mass peak is 4327.9 corresponding to Aβ1-40 (expected m/z 4329.9).

tions with apoE specific receptors on cell membranes (26). This hypothesis requires the existence of brain sAβ/apoE complexes, which we have demonstrated.

Our studies also make the observation that AD brain sAβ appears to be more dimeric than monomeric. This is in agreement with a recent study using fluorescence resonance energy transfer to examine the structure of soluble Aβ1-40 at low concentrations, showing that under these conditions sAβ peptides can exist as a stable dimer (30). Interestingly apoE in the AD brain appears to be complexed primarily to this dimeric form of sAβ. This is evident in our immunoprecipitation experiments using anti-apoE antibodies in brain supernatants (see figure 3B). The Aβ immunoreactive band coimmunoprecipitating with apoE is 8kDa rather than 4kDa. Conversely, in our immunoprecipitation experiments using pooled normal CSF, sAβ was found to exist mainly as a 4kDa band (see figure 1A). The relative lack of dimeric sAβ in CSF may be related to concentration and/or the known anti-fibrillogenic properties of CSF (31). The complexing of apoE primarily to dimeric brain sAβ is consistent with our previously stated hypothesis that apoE acts as a chaperone to Aβ, influencing its conformational state (32,33). We do not know if this dimeric form of AD brain sAβ is abnormal since the quantity was too small for conformational analysis; however, we have previously shown that apoE preferentially binds Aβ peptides in a β-pleated sheet conformation (25), suggesting that the dimeric sAβ which we found in AD brain supernatants may represent an intermediate between normal sAβ and Aβ peptides deposited in brain parenchyma.

The presence of the apoE4 allele is the major identified risk for the presence of late-onset AD. This report provides the first evidence for the existence of brain sAβ-apoE complexes. Another important gene for the presence of AD is presenilin 1 (PS1) ; mutations of this gene are the most common association with early-onset AD (34). Like apoE, PS1 has been found to be component of neuritic plaques (35,36). It remains to be determined if each of the proteins which have been linked to the development of AD can also interact with sAβ to influence amyloid deposition.

ACKNOWLEDGMENTS

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BRAIN UPTAKE OF CIRCULATING APOLIPOPROTEINS J AND E COMPLEXED TO ALZHEIMER'S AMYLOID 8 [1]

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Amyloid B (AB) is a fibrillar component in Alzheimer's disease amyloid deposits and a soluble peptide (sAfi) normally present in body fluids. We have recently reported that the blood-brain barrier (BBB) has a capability to control cerebrovascular sequestration and transport of circulating sAfi. In this study, we examined whether two circulating amyloid-associated proteins shown to bind sAfi, apolipoproteins J (apo J) and E (apo E), can cross the BBB alone and/or complexed to a synthetic peptide homologous to a major forro of sAB, sAB,_.. Brain perfusion experiments in guinea pigs showed significant uptake of both apo J and sAB, apo J complexes. In contrast, blood-brain transport of sAB, apo E was negligible, while apo E had a limited access across the BBB, indicating that the apo E found within the brain is produced locally. It is concluded that sAB, ., binding to apo J and apo E results in significant (> 100-fold) difference in brain uptake of their respective complexes. We hypothesize that in normal brain apo J facilitates sAB transport.

Amyloid B (AB) is the principal constituent of the fibrillar aggregates of amyloid in senile plaques and cerebral blood vessels in Alzheimer's disease (AD) and related disorders (1-4). The AB extracted from senile plaques of AD patients contains 42/44 amino acids (1-4), while the vascular amyloid is shorter at the C-terminus, i.e., 39/40 amino acids long (5). Recently, soluble AB like peptides (sAB), with major form similar to vascular amyloid, AB_{1-40}, and minor $AB_{,\_42}$

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The abbreviations used are: BBB, blood-brain barrier; AB, amyloid B; sAB, soluble amyloid 13; apo E, apolipoprotein E; apo J, apolipoprotein J; AD, Alzheimer's Disease; CNS, central nervous system.

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sequences, were identified in media from cell cultures of untransfected and B-amyloid precursc:, protein-transfected celis, as well as in cerebrospinal fluid (CSF) and plasma from normal and AD patients (6-10). The discovery of circulating sAB suggests that AD and systemic amyloidosis may have unifying features (11). This hypothesis has been supported by our recent findings that sAB,-40 crosses the blood-brain barrier (BBB) (12). The putative role of certain apolipoproteins in controlling levels of sAB in the extracellular and intracellular fluids of brain and other tissues, as well as their influence on fibril formation has been considered (11). It was shown that sAB specifically binds to apolipoprotein J (apo J) (13) as well as to two different isoforms of apolipoprotein E (E3 and E4) (14). The complex sAB, $_{40}$-apo J was demonstrated in normal CSF (13), and our recent in vivo studies indicate that in normal human plasma sAB is chieflv transported with apo J as a component of the high density lipoproteins (HDL) (15). On the othe hand, it has been suggested that apo E in brain acts as a pathological chaperone by modulating B-pleated conformation of AB (11). Consistent with this theory, there is genetic linkage between apo E4 and late onset AD (14). In this study, we examined the BBB transport and sequestration of apo J and apo E alone or complexed with a synthetic peptide homologous to major form of sAB, $_{sAB,-40}$. These experiments were performed to test the hypothesis that circulating apolipoproteins may be involved in the control of sAB homeostasis in the brain and cerebral microvessels.

METHODS

Vascular brain perfusion - Guinea-pigs (Hartley strain) of either sex and 250 to 300 g body weight were anesthetized intramuscularly with 6 mg/kg xylazine (Rompun; Mobay, KS) and 30 mg/kg ketamine (Ketacet; Aveco, IA) prior to surgical exposure of the neck vessels. Details of the vascular brain perfusion technique were previously described (16,17).

Synthetic peptide and proteins - Peptide sAB$_{_,0}$ was synthesized at the W. M. Keck Facility at Yale University. Human apo J was from Quidel (San Diego, CA); human apo E was from Chemicon International (Temecula, CA). sAB$_{_,0}$, apo J and apo E were labeled with Na[^{125}I] (13.7 mCi/μg, Amersham, Arlington Heights, II) using Iodobeds (Pierce, Rockford, II) following manufacturer's instructions. The specific activities of [^{125}I]AB$_{,-40}$, [^{125}I]apo J, and [^{125}I]apo E were 7.1, 4.8 and 8.9 μCi/μg, respectively. [^{125}1]-labeled sAB$_{1-40}$apo J and sAB$_{1-40}$-apo E complexes were obtained by incubating either iodinated apo J or apo E with sAB $_{1-40}$ in phosphate buffer, pH 7.4 at 37°C for 3 hr. The specific activities of iodinated sAB, $_{-40}$apo J and sAB $_{1-40}$-apo E complexes were 4.4 and 8.5 μCi/μg, respectively. The formation of complexes was assessed on 1% agarose gels (Paragon, Beckman, Fullerton, CA) and visualized via Coomassie blue (18).

Brain uptake measurements - Radioiodinated sAB $_{1-40}$, apo J, apo E, sAB$_{1-40}$apo J and sAB$_{_,0}$ apo E were introduced separately into the perfusion circuit by a Harvard slow-drive syringe punir at a rate of 0.2 ml/min within 10 min. [^{14}C]sucrose was used as a standard cerebrovascular space marker. [^{125}I]apo J and [^{125}I]apo E were also studied in the presence of unlabeled apo J and/or apo E. Unlabeled apo J and apo E were pre-infused into the brain 5 min before perfusion of [^{125}1]-labeled ligand, and then for additional 10 min simultaneously with labeled ligand. In all experiments, the perfusion was terminated by severing the right common carotid artery and decapitating the animal. The ipsilateral cerebral cortex was quickly removed from the skull, arachnoid membranes were peeled away, and the choroid plexus was discarded. The brain was

then homogenized, and [^{125}I] and [^{14}C] radioactivities determined in a gamma counter Beckman 4000 and Beckman LS-7500 liquid scintillation spectrometer, respectively. In some experiments, following 10 min perfusion the brain tissue was subjected to the capillary depletion procedure and microvessels were separated from the perfused brain (19,20), to determine tightly bound microvascular fraction and the transendothelial transport, i.e., the amount of test-ligand that has crossed the BBB into brain parenchyma.

Calculations - Brain uptake was expressed as volume of distribution (V_D) of radiolabeled sAB_{I-40}, apo J, apo E, $sAB_{,-40}$-apo J, $sAB_{,-40}$-apo E and sucrose in the brain calculated as (16,19): $V_D = C_{BR}/C_{PL}$ (eq. 1), where C_{BR} and C_{PL} are DPM or CMP/g of brain, and DPM or CPM/ml of perfusate, for [^{125}I] or [^{14}C] radioactivities. Correction for the test peptide/protein isotopic uptake within brain vascular space was made by subtracting brain uptake of cerebrovascular space marker sucrose as (17,19): $V_D$test - $V_D$sucrose (eq. 2). During perfusion, the concentration of test tracers in the arterial inflow, C_{PL}, remains constant. When brain tissue was subjected to the capillary-depletion step, V_D values for microvessels and capillary-depleted brains were calculated by eq. 2, and DPM or CPM for tracers were expressed per unit mass of microvascular and capillary-depleted brain tissue, respectively. Results were compared by analysis of variance and Student's t test; $p < 0.05$ was taken to be statistically significant.

RESULTS

Table 1 illustrates significant brain uptake of circulating radioiodinated apo J and sAB_{I-40} apo J complexes within only 10 min of arterial infusion; their respective V_D values were 16 and 37-fold higher than the uptake of sucrose. In contrast, V_D value for apo E was only about 2-fold higher than for sucrose, while brain uptake of sAB_{I-40}-apo E complexes was even lower relative to sucrose. Uptake of sAB_{I-40}-apo J complexes was significantly higher that uptake of sABI-40 alone, apo J alone, and sAB_{I-40}-apo E complexes by about 4, 2 and more than 100-fold, respectively. On the other hand, uptake of sAB_{I-40} apo E complexes was significantly lower in comparison to sAB_{I-40} and apo E alone, by about 30 and 7-fold, respectively. As shown in Fig.

TABLE 1. BRAIN UPTAKE, V_D, OF IODINATED $_{sABI-40}$ AND APOLIPPROTEINS J AND E COMPLEXED TO $_{sAB,-40}$ AFTER 10 MIN OF CAROTID ARTERIAL INFUSION

TRACER	V_D (µl/9)	RI	n
sAB_{I-40}	26.8 +3.12	9.71	19
sAB_{I-40} apo J	102.5 +32.7[a]	37.13	4
sAB_{I-40} apo E	0.91 +0.32[b]	0.32	6
apo J	43.4 + 10.0[c]	15.71	8
apo E	6.53 ± 3.20[d]	2.34	6
sucrose	2.81 +0.80	1.00	12

Values are means ± SE, n is number of perfused brains. RI, relative increase; entries are ratios of V_D peptide/protein vs. V_D sucrose. V_D for all peptide/protein (except $sAB_{,-40}$-apo E) are signifcantly higher than for sucrose. [a] $p < 0.002$ for sAB_{I-41}-apo J vs. $sAB_{,-40}$ and $sAB_{,-4}$,-apo E by ANOVA; [b] $p < 0.002$ for $sAB_{,-40}$apo E vs. sAS_{I-40} by Student's t-test; [c] $p < 0.05$ for apo J vs. $sAB_{,-40}$apo J by Student's t-test; [d] $p < 0.05$ for apo E vs. sAB_{I-40} apo E by Student's t-test.

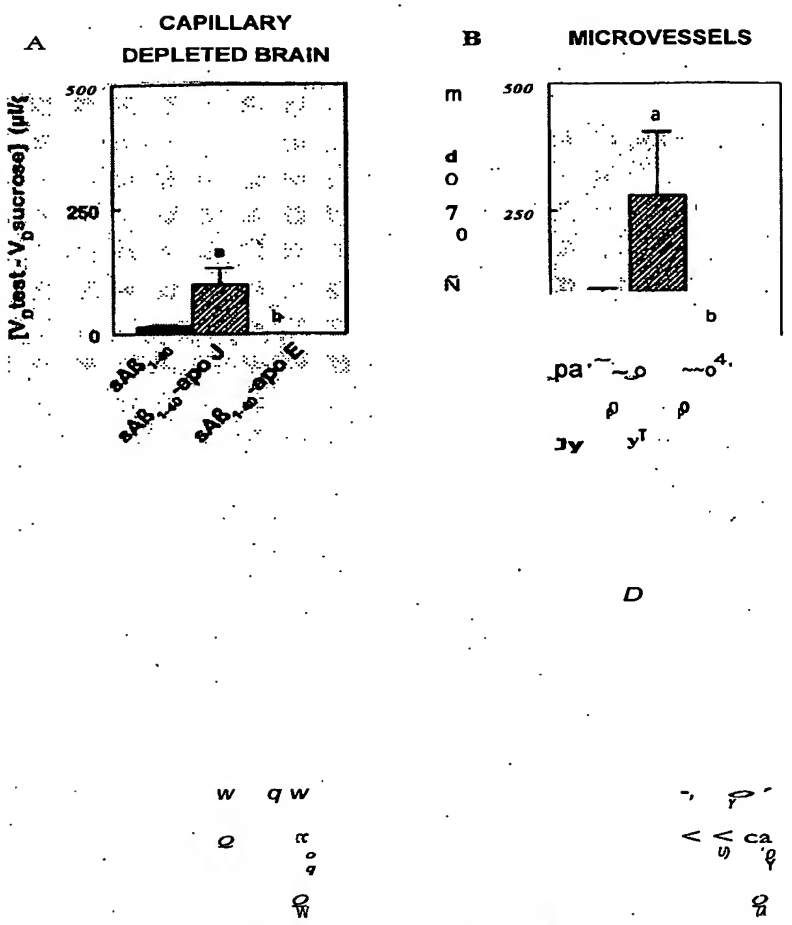

Figure1. **Compartmental distribution of radioiodinated sAB 10, sAB, apo J and sAB, apo E following 10 min of brain perfusion in the guinea-pig.** A, transport of test-tracers into brain parenchyma. B, sequestration of test-tracers by cerebral microvessels. Values are mean ±.SE, n = 4-8. ap < 0.02 to 0.001 for sAB,_,--apo J vs. sAB,... and sAB,__-apo E by ANOVA; [b]p < 0.02 to 0.01 for sAI3,_, _apo E vs. sAf3..._ by Student's t test. C and D, electrophoretic mobility of sAf3.__, apo J, apo E and the corresponding complexes, assessed by agarose gel electrophoresis. Five micrograms of protein were loaded in each lane. Staining was Coomassie blue.

l A, the transendothelial transport of sA13.__--apo E complexes is zero, and their sequestration by microvessels is barely aboye background level (Fig. 1B). In contrast, both transport into brain parenchyma and binding to cerebral microvessels of sAf3 .__-apo J complexes were significantly higher relative to sA13.__ alone (Figs. lA and B). The complex formation has been assessed in non-denaturing conditions on agarose gels (Figs. 1C and 2D), and by non-SDS-PAGE (not shown). Fig. 2 illustrates almost complete self-inhibition of [^{25}1]-apo J brain uptake, transendothelial transport and microvascular binding by low concentrations of unlabeled apo J.

On the other hand, low brain extraction, transport and microvascular binding of [^{125}I]-apo E was non-inhibitable by low nM concentration of unlabeled apo E (Fig. 2).

DISCUSSION

This study has demonstrated that binding of sAI3 ... to apo J and apo E results in significant (> 100-fold) difference in brain uptake of their circulating complexes, ranging from remarkable values as determined for sAB...apo J complex, to negligible and almost unmeasurable values as in a case of sAB ...-apo E complex (Table 1). Compartmental brain analysis has revealed the transendothelial transport and extremely high degree of capillary sequestration of sAB...apo J (Fig. 2), in contrast to sAB,...apo E. After only 10 min of carotid arterial infusions, brain uptake of radioiodinated sAB ... and sAB,...apo J reached about 2.6% and 10.2% of their respective concentrations in plasma perfusate, that was about 10 and 37-fold higher than the uptake of cerebrovascular space marker sucrose. In contrast to present findings, relatively low BBB permeability to human sAB... has been recently reported in mice (21). The discrepancy with this study might be explained by the species difference, and/or by the difference in the peptide sequence. Namely, the analogous AB... in mouse and rat differs at three positions [Gly^{5}, Phe^{10}, Arg^{13}] (22) from human AB, while the amino acid sequences of AB in guinea-pigs and humans are identical (23).

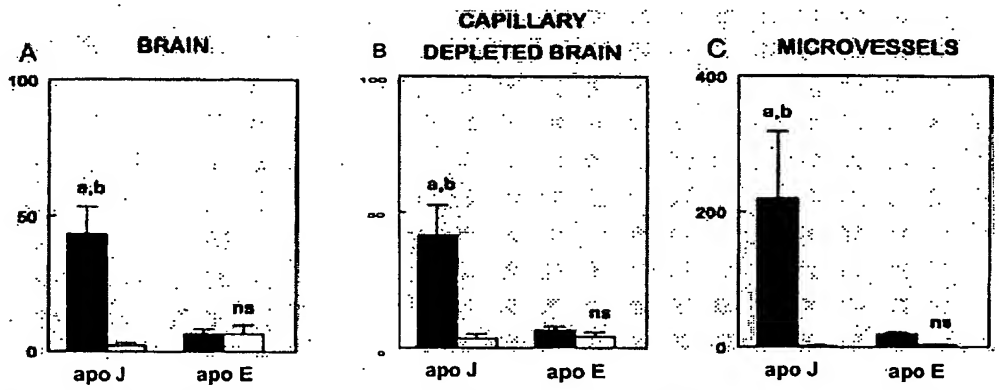

Figure 2. **Brain uptake and compartmental distribution of radioiodinated apo J and apo E following 10 min of brain perfusion in the guinea-pig.** A, blood-to-brain transport of test-tracers. B, transport of test-tracers into brain parenchyma. C, sequestration of test-tracers by cerebral microvessels. Values are mean ± SE, n = 3-8. Solid bars represent distributions of [^{125}I]apo J and [^{125}I]apo E in the absence of unlabeled apolipoproteins. Open bars represent distribution of [^{125}I]apo J in the presence of 44 nM unlabeled apo J, and distribution of [^{125}I]apo E in the presence of 6 nM unlabeled apo E. $^{a}p < 0.05$ to 0.005 for apo J self-inhibition; -, non-significant for apo E self-inhibition; $^{b}p < 0.05$ to 0.003 for [^{125}I]apo J vs. [^{125}I]apo E, by Student's t-test.

Although, knowledge of the mechanisms by which peptides and proteins are transported across the BBB is still limited, recent reviews (17,24-27) favored the theory that several peptides and proteins are transported across cerebral capillaries by specific receptor and/or carrier-mediated transcytosis. According to our recent report, it seems that this is also true for $sAf3_{1-40}$ (12). The finding that normal BBB has a capability to sequester and transport sAf3 and sAf3-apo J complexes (Table 1, Fig. 2), would imply that both processes may contribute to accumulation of circulating sAl3 in the extracellular space of brain and cerebral vessels. The possibility that in normal brain apo J may have a role of carrier protein across the BBB for sAB, is also supported by our recent observation that in the plasma sAl3 is preferentially transported as a complex with apo J incorporated within HDL_3 and VHDL (15). On the other hand, sAB complexed to apc ' will have minimal permeability to the BBB. Since apo E has limited access across the BBB, .. is likely that the apo E found within normal central nervous system (CNS) is produced locally. This is consistent with the distinct sialyation pattern of apo E in the CNS (28). Our studies suggest that apo J may have systemic origin. However, in the disease state it is unclear what the source of the apo E is, since the BBB permeability could be altered to apo E. Apo E is known to be an acute-phase reactant within the CNS, and in response to injury apo E may assume the role of pathological chaperone by binding and sequestering sAf3 within the CNS (29).

The mechanisms by which apo J and apo E influence BBB trafficking of sAf3 deserve to be explored in greater detall. Significant brain uptake of apo J (Table 1), i.e., 3.5 to 4.5-fold higher than for insulin (24), as well as inhibition of [^{125}1]-apo J in situ BBB binding and transendothelial transport by low nM concentrations of unlabeled apo J (Fig 2) (in a do.-:-dependent fashion, data not shown), would suggest the presence of a specific high affinity receptor and/or carrier for apo J. The possibility that a lower affinity BBB binding and/or transport mechanism(s) operates for apo E could not be ruled out based on present data. In this study, human apo E containing mainly apo E3 isoform was used. Our preliminary experiments utilizing human recombinant apo E3 and apo E4 suggested similar low BBB uptake. In summary, present results provide initial experimental evidence in support of the hypothesis that circulating apo J and apo E may be involved in controlling transport of sAB in normal brain. However, it remains to be determined whether BBB transport and microvascular sequestration of these apolipoproteins alone or complexed with sAl3 are critical for the development of A 1) pathology.

ACKNOWLEDGMENTS

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Short Communication

Acceleration of Alzheimer's Fibril Formation by Apolipoprotein E *In Vitro*

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Numerous studies have established a linkage between the apolipoprotein (apo) E4 allele and late-onset Alzheimer's disease. It remains unclear if apo E plays a direct role in the pathogenesis of Alzheimer's disease and what, if any, are its significant interactions with amyloid β (Aβ) and tau. Apo E has been found immunohistochemically in all types of amyloid deposits and apo E fragments have been isolated from amyloid. Furthermore, apo E has been shown to bind soluble Aβ. It has been proposed that apo E acts to promote and/or modulate Aβ fibril formation. It is well established that peptides homologous to Aβ will form amyloid-like fibrils in solution. With the use of electron microscopy and a thioflavin T assay for fibril formation we found that apo E and apo E4 in particular enhance this spontaneous fibrillogenesis of Aβ peptides under the in vitro conditions used. These in vitro data suggest that the apo E4 isoform is a risk factor for Alzheimer's disease that acts to accelerate a process that can occur in its absence. (Am J Pathol 1994, 145:1030–1035)

Recent data have shown linkage between the apolipoprotein (apo)E allele, ε4, and late-onset Alzheimer's disease (AD; for review see ref. 1). This linkage has been confirmed by numerous investigators and in different ethnic populations.[1] Furthermore, the inheritance of the apo E4 allele has been associated with an earlier age of onset and a greater amyloid β (Aβ) immunoreactivity in an allele-dose-dependent manner.[1–3] Apo E is a 34-kda glycosylated protein encoded by a four-exon gene on the long arm of chromosome 19, and is present in both plasma and cerebrospinal fluid.[4,5] The three major isoforms of apo E are apo E2, E3, and E4. 60% of individuals are homozygous for apo E3, while 2 to 3% are E4 homozygous. Apo E4 differs from E3 by a single amino acid residue; E4 has an arginine at residue 112 instead of cysteine. Apo E2 has cysteines at both residues 112 and 158.[4] Of all the apolipoproteins apo E is the most important for nervous tissue, where it is critical in the mobilization and redistribution of cholesterol during normal growth and following injury.[6–8] In the nervous system apo E is an acute-phase reactant in that its levels rise following both peripheral and central nervous system (CNS) injury.[6–8] It is produced within the CNS by both glial cells and macrophages. Neurons do not produce apo E but express the apo E-binding receptor, low-density lipoprotein receptor-related protein by which it can be internalized.[3]

Before the discovery of the linkage between AD and apo E it was found that apo E is present immunohistochemically in the lesions that characterize AD, senile plaques and neurofibrillary tangles (NFT).[9,10] In addition it is found in all other types of amyloid deposits, including prion diseases.[10] Apo E was also known to be closely associated with certain systemic amyloid deposits by direct biochemical analysis (ref. 11 and unpublished observations). These observations and the known other properties of apo E led us to propose that apo E may function as a "pathological chaperone" in amyloid deposits.[10] By this we mean that apo E can promote the aggregation of amyloi-

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dogenic proteins into the β-pleated sheet conformation that is typical of all amyloid deposits. This would in turn inhibit further proteolysis of these deposits.

In this paper we report the use of an *in vitro* model of amyloid fibril formation to test the concept that apo E functions as a promoter of the fibrillogenesis of Aβ peptides. Synthetic peptides homologous to Aβ will spontaneously form amyloid-like fibrils in solution.[12–16] We used peptides homologous to Aβ 1–40, which is the major sequence of the soluble Aβ (sAβ)[17–20] to study the effects of the presence of apo E3 and E4 on the process of fibrillogenesis by means of electron microscopy and a thioflavin T assay. The latter is an established fluorescence assay for the quantitation of amyloid fibril growth in solution.[21–23]

Materials and Methods

Synthetic Peptides and Proteins

Peptide Aβ 1–40, corresponding to residues 1–40 of Aβ, was synthesized by solid-phase procedures at the Center for Analysis and Synthesis of Macromolecules (State University of New York, Stony Brook). Crude peptides were purified by high-performance liquid chromatography using a 3.8 × 300 mm μ-Bondapak C18 column and a linear gradient of 0 to 80% acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 2 ml/min. Peptide sequences were determined by automatic Edman degradation on a 477-A protein sequencer and the PTH derivatives analyzed with an online 120-A PTH analyzer (Applied Biosystems, Foster City, CA). Purity and quantitation of the peptides were done by amino acid analysis using a Pico-Tag analyzer (Waters Chromatography Division, Marlboro, MA).

Recombinant apo E3 and E4 were purchased from Calbiochem Corp. (San Diego, CA) and also produced as described.[24] The purity of the apo E preparation was >95% as assessed by SDS-PAGE and amino terminal sequencing as described above. Bovine ubiquitin and bovine serum albumin (BSA) were purchased from Sigma Chemical Co. (St. Louis, MO). Protein content of these preparations was determined using a micro BCA protein assay reagent kit (Pierce Chemical Co., Rockford, IL).

Aggregation and Fluorometric Experiments

A stock solution of Aβ 1–40 was prepared in 0.1% trifluoroacetic acid, 50% acetonitrile, and stored at −20 C, with the concentration determined by amino acid analysis. Aliquots of this stock were lyophilized, and incubation was started at room temperature by adding 0.1 mol/L Tris pH 7.4. Stock solution of recombinant apo E was made in 0.1 mol/L ammonium bicarbonate with the protein content determined using a BCA protein assay kit. Aliquots were lyophilized and resuspended in 0.1 mol/L Tris pH 7.4. The studies were done at a concentration of 250 μmol/L of Aβ 1–40 and 2.5 μmol/L apo E3 or apo E4. The incubation volume was 35λ. Aβ 1–40 was incubated alone or with recombinant apo E3 or E4 at room temperature for the indicated intervals.

Aβ incubations with bovine ubiquitin and BSA were done as a control. Apo E3 and E4, BSA and ubiquitin were also incubated alone as a control. For the latter control experiments apo E3 and E4 were incubated at a concentration of 10 μmol/L and BSA and ubiquitin at 500 μmol/L.

Fluorometry was performed as described.[21,23] The incubated samples were added to 50 mmol/L glycine pH 9.2, 2 μmol/L thioflavin T (Sigma Chemical Co.) in a final volume of 2 ml. Fluorescence was measured at excitation 435 nm and emission 485 nm in a Hitachi F-2000 fluorescence spectrophotometer. A time scan of fluorescence was performed and three values after the decay reached a plateau (280, 290, and 300 seconds) were averaged after subtracting the background fluorescence of 2.0 μmol/L thioflavin T. Samples were run in duplicate. The mean ± SD, for three separate experiments, is plotted in Figure 1.

Electron Microscopy

Aβ peptides alone or incubated with recombinant apo E3 and E4 and control proteins at the concentrations used above were incubated for 7 days at room temperature and placed on carbon formar-coated 400-mesh, fresh glow-discharged nickel grids (Ladd Research Industries Inc., Burlington, VT) for 30 seconds blotted and stained with 2% uranyl acetate (Ladd Research Industries Inc.) for 30 seconds, under a vapor of 2% glutaraldehyde. Grids were visualized on a Zeiss EM 10 electron microscope at 80 kV.

Results

Freshly suspended Aβ 1–40 either alone or co-incubated with apo E in the fluorescence buffer showed no specific thioflavin-T fluorescence at emission 485 nm. During the 10 days of incubation there was a gradual increase of the fluorescence of both Aβ alone, and Aβ co-incubated with apo E3 and E4 (Figure 1). However, the rate of increase was greatest with

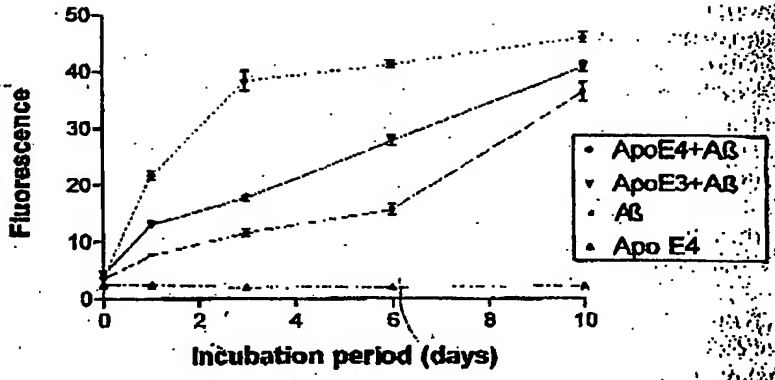

Figure 1. *Thioflavin T assay of Aβ 1–40 amyloid fibril formation. The mean fluorescence ± standard deviation (in arbitrary units) is plotted against the incubation period in days of: Aβ 1–40 and apo E 4 (◆, connected by short dashes), Aβ 1–40 and apo E3 (▼, connected by straight lines), Aβ 1–40 alone (■, connected by long dashes) and apo E4 alone (▲, connected by long and short dashes).*

Aβ 1–40 co-incubated with apo E4. Apo E3 or E4 alone showed no increase in fluorescence over this time period. Aβ 1–40 incubated with BSA or ubiquitin gave similar fluorescence to the Aβ 1–40 alone (data not shown). The differences between Aβ 1–40 alone and Aβ with apo E3 or E4 were greatest in the first few days of incubation and were reduced by day 10, suggesting that the apo E was acting to accelerate or seed fibril formation.

By electron microscopy both Aβ peptides alone and those incubated with apo E3 or E4 formed amyloid-like fibrils. Aβ peptides alone formed fewer fibrils (Figure 2A) than Aβ with either apo E3 or E4. These were ~10 nm in width, unbranched, and up to 2 μm in length. Aβ peptides with apo E4 revealed a larger number of variable-length fibrils, which tended to produce a dense matrix-like meshwork of 7- to 10-nm fibrils (Figure 2B); fewer fibrils were present with Aβ and apo E3. No fibrils were noted with apo E3 or E4 incubated alone. This electron microscopic appearance is similar to another report where Aβ peptides fibril formation was studied in the presence of plasma-purified apo E3 and E4.[25]

Discussion

Extensive evidence for the linkage between late-onset AD and the apo E4 allele has been reported.[1,26] However, linkage does not always provide easy clues to pathogenesis. For example, the linkage between HLA-B27 and ankylosing spondylitis has been known for several years and is stronger than the linkage between apo E and AD; nevertheless, the mechanism remains unknown.[27] In the case of apo E direct involvement in amyloidogenesis is suggested by a number of observations. Apo E has been found in all types of amyloid deposits immunohistochemically,[10,49] and apo E fragments have been found by sequencing in a number of biochemically distinct amyloid deposits (ref. 11 and unpublished observations). Furthermore, sAβ has been shown to bind apo E by a number of different methods.[1] A number of different roles for apo E have been suggested in the pathogenesis of AD, such as an interaction with tau. The binding of apo E3 to tau may prevent it from hyperphosphorylation, hence inhibiting NFT formation.[28] In this hypothesis it is the lack of apo E3 or E2 rather than the presence of E4 that is the important factor. This theory is difficult to reconcile with observations such as: the lack of correlation of apo E isotypes with other conditions where NFT develops;[29] the presence of correlation between apo E4 and the Lewy body variant of AD,[30] where NFT formation is absent or minimal; and studies on the aged rhesus monkey model of AD.[31] Despite these shortcomings the theory that apo E3 acts to prevent NFTs raised the possibility that part of the linkage of AD with apo E4 may be due to the lack of a protective factor. One such protective factor could be apo E2. This is suggested by the negative association between AD and apo E2, as well as the association between longevity and apo E2.[32] An alternative theory is that the apo E4 isoform may be less efficient in the pivotal role apo E plays during the CNS mobilization and distribution of cholesterol following injury.[4] Apo E-containing lipoproteins have an increasing binding to low-density lipoprotein receptors from apo E2 to apo E3 to apo E4.[4] As a result the presence of apo E4 alters the transport of cholesterol and phospholipids. This could lead to defective reinnervation and poor synaptic plasticity. In agreement with this theory recent preliminary experiments have shown that in the presence of β migrating, very low-density lipoprotein apo E3 increases neurite outgrowth, whereas apo E4 decreases outgrowth in tissue culture.[33,34] Both our electron microscopic and thioflavin T assay data suggest that apo E, and apo E4 in particular, promote the known fibrillogenic potential of Aβ peptides at the stated concentrations

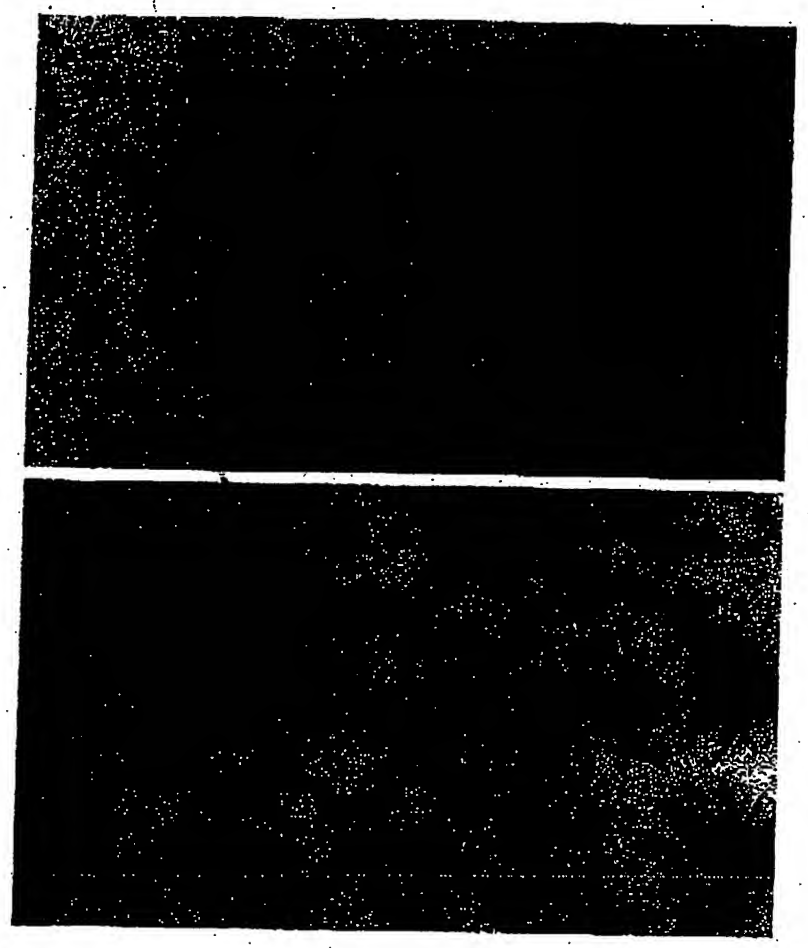

Figure 2. *Electron micrographs of negatively stained Aβ 1–40 fibrils (a) and Aβ 1–40 incubated with apo E4 (b). The scale bar represents 1000 Å.*

and conditions used in our experiments. These electron microscopic observations are similar to those found in two other papers,[25,44] where Aβ peptides formed a greater number of fibrils, more quickly in the presence of apo E4. The concentrations used in these *in vitro* studies are much higher than those found in biological fluids and were chosen because they are optimal for fibril formation and may more closely match conditions found in the pathological state. At more physiological concentrations the interactions between apo E and sAβ are likely to be very different. In cerebrospinal fluid Aβ is in the high picomolar range[18] and the concentration of apo E is ~100 to 500 nmol/L.[35] This ratio of Aβ to apo E is the opposite of what we used in our fibril experiments, but in extracted amyloid the ratio of the amyloid protein to apo E is ~100:1 (unpublished observations); hence this ratio was used. We hypothesize that other initial factors lead to increased local concentrations of Aβ peptides and its deposition first as preamyloid in the brain. In response to this deposition numerous proteins are up-regulated including apo E. Apo E levels are known to rise several hundredfold in response to peripheral nerve damage.[7] In the CNS apo E levels have also been shown to rise in response to injury.[6,8] For example, in the compensatory response of the hippocampal formation to entorhinnal cortex lesions marked increased expression of apo E has been shown in the differentiated zone of the molecular layer.[8] Hence, in pathological conditions where local Aβ levels are high and apo E is also elevated such as within plaques, the fibril-promoting effect of apo E4, which we document *in vitro*, is also likely to be important *in vivo*. Since the process of amyloid formation is thought to proceed over decades even a slight promoting effect will be significant over time. The interaction between Aβ and apo E in plaques may also be dependent on other factors such as proteoglycans, which are amyloid-associated proteins[36] that apo E is known to bind.[37]

Our *in vitro* data indicate that apo E, in the presence of high concentrations of Aβ peptides, acts to accelerate a process that can take place in its absence. The latter is consistent with the epidemiological observation that some 40% of patients with AD do not possess an ε4 allele; hence, apo E is neither necessary nor sufficient to cause the disease.[38] This is similar to the mechanism of action of some of the known βPP mutations. For example, Aβ peptides that contain the mutation found in the Dutch variant of early onset AD,[39] also known as hereditary cerebral hemorrhage with amyloidosis, Dutch type, form amyloid-like fibril at an accelerated rate[40,41] analogous to wild-type peptides in the presence of apo E. In both the Dutch pedigrees[45] and among early-onset familial AD patients with linkage to chromosome[14,42] no association between the Apo E4 isotype and disease expression has been found. In these families another gene product is the major accelerator of AD pathology. The apo E4 allele can be viewed as one of several possible risk factors that can predispose to the polygenetic and multifactorial illness of AD.[1] The discovery of linkage between the apo E4 allele and AD and the interaction between apo E isoforms and Aβ, like the finding of βPP mutations, provides a starting point for the investigation of the central events that mediate disease progression in AD.

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